Genetic variation in oil, growth and propagation traits of *Backhousia citriodora* (F. Muell), and implications for breeding strategy

Wenbin Chen

Department of Forestry
The Australian National University

A thesis submitted for the degree of Master of Science at
The Australian National University

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DECLARATION

The work described in this thesis has been carried out and composed by the undersigned at the Department of Forestry in The Australian National University, Canberra, Australian Capital Territory, Australia. Any assistance received is acknowledged.

Candidate:_____

Supervisor:_____

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ABSTRACT

Backhousia citriodora F. Muell is a tree species which occurs naturally as disjunct populations in the tropical and subtropical rainforests of coastal and adjacent upland Queensland, Australia. The citral (aliphatic aldehyde C_{10} H_{16} O) in *B. citriodora* essential oil is of high economic value for a range of uses. Production of high-quality planting stock is urgently required to meet the demand of an embryonic plantation industry. However, no genetic information has been available for this species. This thesis reports an investigation of variation in oil, growth and propagation traits of *B. citriodora*, genetic parameters for these traits, and their implications for breeding and propagation strategies.

The research was based on two trials established in South East Queensland in 1995 and 1996: Trial 1, a progeny test of 16 families representing four provenances; Trial 2, a clonal test for the assessment of propagation traits, using cuttings from Trial 1. The experimental population yielded, on average, leaf-oil in sufficient quantity (2.6% of fresh leaf; 4.5% of dry leaf) and suitable quality (85% citral) for use as commercial source of *B. citriodora* oil. Variance analysis suggested that there were significant differences between and within families in most traits of interest. In terms of predicted breeding values, family 1016 ranked as best for total citral, leaf biomass and coppicing ability, while families 1029 and 1030 were identified as the best for rooting success and seedling survival, respectively. The Eumundi population was superior for all traits other than rooting success, the Woondum population was superior for rooting success, and the Noosa National Park population was best for seedling survival.

Narrow-sense heritabilities were moderate to high (0.2 to 1) for oil traits - with the exception of those for concentrations of neral, geranial and citral - and for growth and propagation traits assessed in Trial 1. These estimates were not precise due to the relatively small data set. Broad-sense heritability estimates for propagation traits in Trial 2 were moderate. Genetic correlations between most oil and growth traits were large and positive. Survival had very weak correlations with all traits and can be considered as an independent trait. There were negative correlations associated with rooting success, which may impose a constraint on the options for deploying *B. citriodora*.

A breeding strategy was developed to address three objectives: immediate deployment of selected materials, long-term breeding for oil and growth traits, and joint improvement of rooting success and total citral. The strategy assumes that cuttings will be used as the primary means of multiplication, and evidence suggests that improvement in propagation success will follow the development of better cultivation technologies. However, as *B. citriodora* flowers after only 18 months, a seedling deployment strategy would also deliver rapid gains.

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Although my studies in Australia are over, I have for ever happy memories of the beautiful country, the nice University and the friendly people in Australia.

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CHAPTER 1. INTRODUCTION

Backhousia citriodora F. Muell., a tree species valued for essential oil production, occurs naturally in disjunct populations in the tropical and subtropical rain forests of coastal and upland south eastern Queensland, Australia. Leaves of *B. citriodora* yield an oil containing over 90 percent citral (aliphatic aldehyde $C_{10}H_{16}O$) (Francis, 1981), which is of high economic value for a range of uses in perfumes, food and beverage, and aromatherapy. Early in this century, industries exploiting *B. citriodora* oil prospered, but they subsequently declined. There has been a recent resurgence of interest in *B. citriodora* oil, resulting in part from the following concerns.

1.1 World forest resources and sustainable development

Forests, covering nearly 30 percent of the earth's total land area, are a valuable environmental and economic resource for supporting natural systems and improving human welfare (Sharma, 1992). They provide timber and many other economic and environmental goods and services (Rowe *et al.*, 1992).

The total growing stock of the world's forests, approximately 373 billion m^3, presently produces some 11 billion m^3 of wood annually (Sharma *et al.*, 1992). While the current demand for wood is about 4.4 billion m^3 per annum, demand may increase by 50 percent, to 6.6 billion m^3, by the year 2025 (Sharma *et al.*, 1992). Concurrently, the world's human population is expected to increase, from approximately 5.4 billion in the late 1990s to 9.6 billion by the year 2050 (Bos *et al.*, 1994), and less financial resources per capita will be available to manage and sustain forest populations (Dvorak, 1996). The increasing demand for wood products is likely to be accompanied by increased demand for non-wood products such as "green foods", essential oils and environmental services. Clearly, the forest resource should be managed to

continue serving the increasing needs of the people and to enhance their social development.

Unfortunately, destructive human activities in the past have caused loss of about one-third of the world's forests (Sharma *et al.*, 1992). Currently, deforestation in the tropics is estimated to be occurring at a rate of 17 to 20 million hectares annually (Rowe *et al.*, 1992). One to three billion metric tons of CO_2 are released into the atmosphere each year from burning associated with deforestation. This CO_2 contributes greatly to global warming, which may be proceeding at a rate of 0.3 - 1 °C per decade (Woodwell, 1992). Deforestation may be responsible for loss of 5 to 15% of the world's total species between 1990 and 2020. Losses of this magnitude would account for 15,000 to 50,000 species per year, corresponding to 50 to 150 species a day (Botkin and Talbot, 1992), and consequently threaten the sustainability of both ecosystems and the supply of forest products.

The consequences of the depletion of the world's forest resources are now widely recognized, and have led to many initiatives to change things for the better. They have focused attention on both sustainable management of forest resources and increased plantation afforestation for various purposes, to address some of the problems of population increase, environmental degradation and resource scarcity.

As declared by the Inter-Ministerial Conference on European Forests at Helsinki in 1993, *sustainable management means the stewardship and use of forest lands in a way, and at a rate, that maintains their biodiversity, productivity, regeneration capacity, vitality and their potential to fulfil now, and in the future, relevant ecological, economic and social functions at local, national, and global levels; and that does not cause damage to other ecosystems* (Burley, 1996). Tree breeders

and forest geneticists have recognized that they should contribute to the aims of this sustainable management (Burley, 1996).

1.2 Tree breeding in support of sustainable development

Meeting the challenge of sustainable development has brought great progress and significant changes in forest genetics and tree breeding as well as in other forest disciplines (Kanowski, 1993). The principal challenge is that demands from forests for social, environmental and financial benefits will increase as human populations expand their numbers, diversity and cultural needs while the availability of productive land for forestry declines. Tree breeders will have a major role in developing genotypes that can yield the range of benefits required from a growing number of species, on a range of increasingly challenging sites, and with a number of different management systems (Burley, 1996).

Another great challenge will be to explore, evaluate, improve and utilize many hitherto unresearched tropical species that may have significant potential to meet forestry's needs at a time when resources for such domestication appear to be declining (Burley, 1996).

To approach these new challenges, forest geneticists and tree breeders have redirected their efforts to those activities which are necessary to realise not only short-term gains, but also economic and genetic efficiency (Kanowski, 1993). As a result, research in support of tree breeding has shifted from classical operations to more advanced practices which are characterised by the integration of knowledge of reproductive biology, population genetics, biotechnology and quantitative genetic theory. Major foci of such integrated tree-breeding practices are the delivery of realised gain and the diversification of breeding objectives (Kanowski, 1993).

In the past, intensive breeding efforts with trees have been concentrated on a relatively few commercially significant species. It is obvious that these efforts are far from sufficient to meet the various present or future demands of forest genetic resources, of both industrial and non-industrial species (Kanowski, 1993). In order to address the global requirements for improved genetic resources, the development of simple, flexible conceptual models and strategic planning of breeding activities in support of sound breeding strategies, directed towards immediate operational success, has become a priority in research.

In practice, careful consideration should be given to the definition of breeding objectives and criteria, population structure, and elements of the breeding cycle - selection, recombination and transformation, genetic testing and multiplication (Kanowski, 1993). At least some of the requisite economic and genetic information are available for longer cultivated industrial species. However, information is still lacking for many non-industrial species (Kanowski, 1993).

1.3 Importance of *Backhousia citriodora*

This study investigates genetic variation in traits related to essential oil production in *Backhousia citriodora* (F. Muell), one of the thousands of tree species of the rainforest in tropical and subtropical Eastern Australia. The distribution and characteristics of the species are described in Chapter 2.

1.3.1 The essential oil of *B. citriodora*

B. citriodora is not one of the more publicised members of the Australian rainforest flora. It is, however, well-known as a species of high value for essential oil production and ornamental use. The oils of the foliage of *B. citriodora* are typically very rich in the commercially valuable essential oil citral, a mixture of neral and geranial (Penfold *et al.*, 1950; Southwell, 1996). The essential oil, strong smelling foliage, and fruits of *B. citriodora* can be widely

used in perfumes, food and drink flavourings, confectionery, herbal teas, aromatherapy and for medicinal purposes (Doran and House, 1996).

Soon after the species was first described by Ferdinand von Mueller in 1859, it attracted attention as a source of citral before being displaced by cheaper substitutes from alternative sources in Asia and Africa (Doran and House 1996). However, increased interest in Australian native species as a source of natural products has rekindled demand for products from *B. citriodora*; commercial plantations have been established, and planting is expected to expand as demand grows for its products (Doran and House, 1996).

As the natural resource of *B. citriodora* is limited and unavailable or uneconomic to exploit, the future market success of the species will depend on cultivated resources. Unfortunately, recent research has confirmed that seed of *B. citriodora* has a very low germination percentage (1 to 4%), despite profuse flowering, and that seedlings often develop slowly and with a creeper habit (J. Doran[1] and D. Lea[2], pers. comm.). Vegetative propagation by rooted cuttings is likely to be the most effective means of mass production of planting stock for commercial plantations (Doran and House, 1996) or other forms of cultivation. At present, the lack of quality planting stock is constraining the development of this promising new industry (Doran and House, 1996). Consequently, further research - beyond the preliminary work already completed (House *et al.*, 1996) - to promote understanding of genetic variation and propagation of the species is essential to develop appropriate breeding and multiplication strategies to support commercial plantation development.

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[1] Dr. J. C. Doran, Australian Tree Seed Centre, CSIRO Forestry and Forest Products, PO Box E4008, Kingston, ACT 2604, Australia.

[2] Dr. D. Lea, Australian Tree Seed Centre, CSIRO Forestry and Forest Products, PO Box E4008, Kingston, ACT 2604, Australia.

1.3.2 The other potential uses of *B. citriodora*

Besides its use for commercial oil production, *B. citriodora* has long been used as an ornamental plant in parks, gardens and public areas in cities, as it produces seasonally abundant flowers and fragrant lemon-scented leaves. In addition, *B. citriodora* may have potential as a species in agroforestry systems. Its leaves can be harvested several times a year, offering good potential for an income stream to farmers. The species can be propagated vegetatively, which would increase its utility in agroforestry and plantation systems. However, the potential of *B. citriodora* for those systems has not yet been exploited.

This project will contribute to our knowledge of the characteristics of *B. citriodora*. It will provide some of the information required for breeders to make informed decisions and best manage the contribution of improved germplasm to forest and farm production systems, as discussed by Simons *et al*. (1994).

1.3.3 The significance of germplasm and biodiversity conservation

Conservation of genetic resources is another important consideration in managing forest resources for sustainable development. It must be viewed in the broader context as one of the stages of use for forest genetic resources, along with exploration, evaluation and utilisation (Burley, 1996). The forest species and populations that show the greatest economic potential, whether measured in fruit or nut production, forage quantity or quality, tonnes of wood per hectare, or disease resistance will be those that will receive priority in conservation efforts in the next century (Dvorak, 1996).

As a valuable native species of tropical forests in eastern Queensland, *B. citriodora* shows not only economic promise, but also significant biodiversity, cultural and aesthetic values. Unfortunately, its natural extent is very limited - due in part, perhaps, to its low sexual reproductivity and its susceptibility to fire (Doran and Lea, pers. comm.) - and it has been reduced by conversion of

forest for agricultural and urban development. There is a reason to believe that, in certain parts of the natural distribution, the genetic resource of this species could be endangered if effective conservation actions are not carried out in the near future. Therefore, the genetic information resulting from this project could be helpful in supporting conservation decisions.

1.4 Project Objectives

1.4.1 Breeding objectives

If *B. citriodora* is to be grown for oil and fresh leaf production on a commercial scale, there are several points to consider to promote success. Firstly, and most importantly, the flavour and efficacy of the oil are crucial if new and profitable culinary and medicinal products are to be produced from the outset (Costin, 1996). To guarantee this, one must first understand the chemical components of the oil and the manner in which they determine the end flavour and efficacy of the oil.

Secondly, the productivity of the plant must be considered. This could be influenced by many factors, including silviculture, although genetic variation in biomass production is likely to be among the most important.

Finally, an effective means of mass production of planting stock for commercial plantations must be developed. This depends primarily on the reproductive features of the species. As there is insufficient genetic information available on these issues, further research is necessary for effective breeding of high quality planting stock of the species.

1.4.2 Objectives of this study

The purpose of this project is to generate basic genetic information for formulating breeding and propagation strategies of *B. citriodora.* The specific objectives of this study are to:

- estimate genetic parameters in oil yield and composition, biomass production, coppicing ability and rooting ability of cuttings;

- assess the implications for breeding and propagation strategies of *B. citriodora*.

CHAPTER 2. LITERATURE REVIEW

2.1 Description of *Backhousia citriodora*

2.1.1 Nomenclature

The genus *Backhousia* is placed within the family Myrtaceae, and comprises eight described (Guymer, 1988) and one undescribed species (Brophy *et al.*, 1995). The described species are listed below:

Table 2.1 Species of *Backhousia*

1. *B. anisata* Vickery
2. *B. angustifolia* F. Muell.
3. *B. bancroftii* F. Muell. Bailey
4. *B. citriodora* F. Muell.
5. *B. hughesii* C. White
6. *B. kingii* Guymer
7. *B. myrtifolia* Hook. & Harvey
8. *B. sciadophora* F. Muell.

B. citriodora, the most promising of these for essential oil production, is known by the common names of lemon ironwood and lemon scented myrtle (Francis, 1981; Wrigley and Fagg, 1996).

2.1.2 Natural distribution

B. citriodora occurs naturally in rain forests of coastal and upland Queensland, between latitudes 17°30' S and 27° S (Doran and House, 1996) and longitudes 145°20' E and 153°10' E (D. Lea, pers. comm.). Altitude varies from 40 m at Eumundi in the south to 700 m at Lead Creek - Silver Valley in far northern Queensland (D. Lea, pers. comm.). Within this wide geographic range, *B. citriodora* is found mostly in small, disjunct populations. However, near

Proserpine (Conway Range), continuous rainforest edge populations are found. Occurrences of the species are mapped in Figure 2.1 and described in Table 2.2.

Figure 2.1 Natural distribution of *B. citriodora*

Source: Doran, pers. comm.

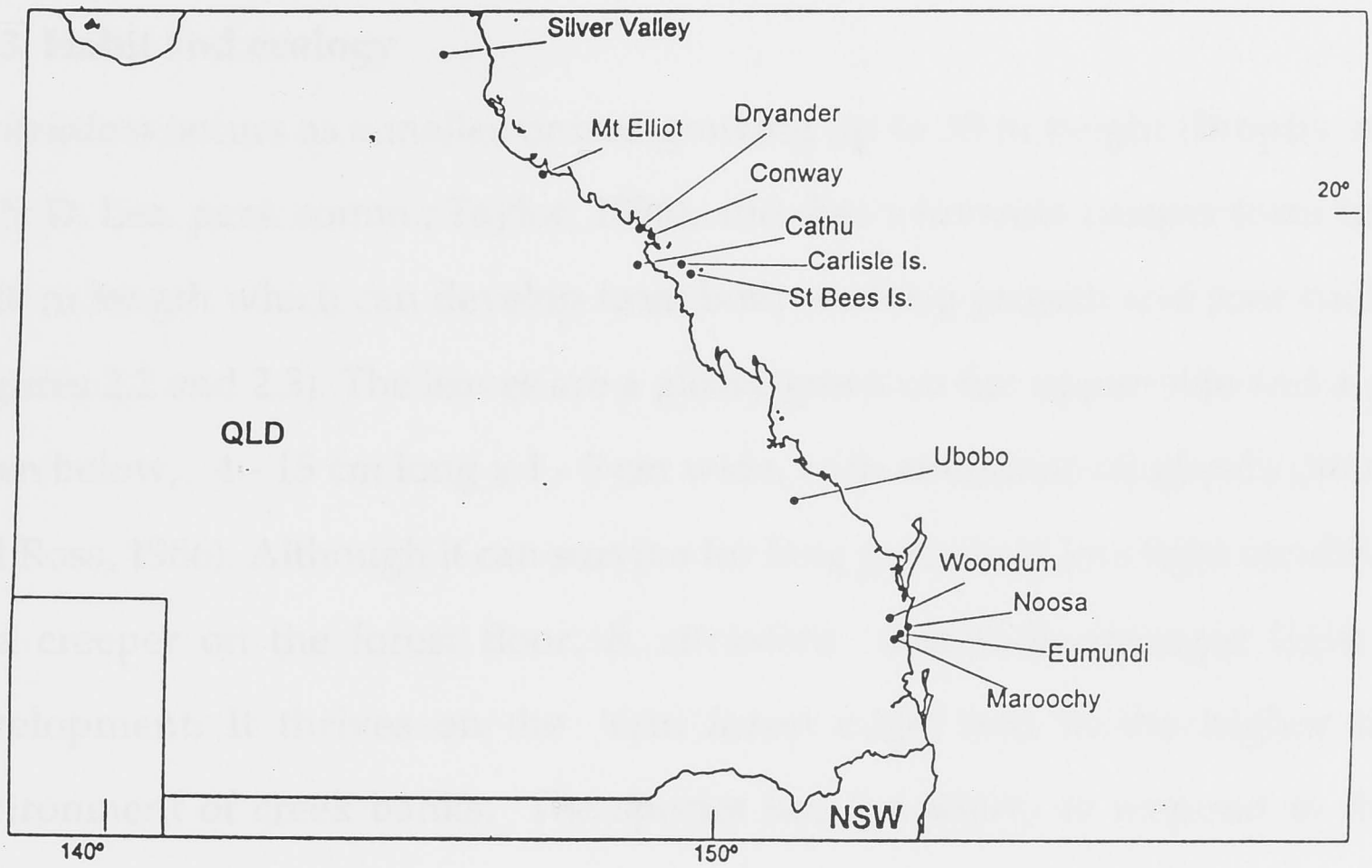


Table 2.2 Natural populations of *B. citriodora*

Location	Lat. (S)	Longit. (E)	Alt. (m)	Form/Ht. (m)
Silver Valley	17°32'	145° 19'	700	mallee to 6
Mt Elliot NP	19° 25'	147° 00'	400	tree to 20
Dryandra NP	20° 16'	148° 34'	100	tree to 30
Conway SF	20° 21'	148° 45'	200	tree to 20
Cathu	20° 47'	148° 34'	200	tree to 20
Carlisle Island	20° 47'	149° 17'	50 to 390	mallee to 4-18
St Bees Island	20° 56'	149° 26'	150	tree to 18
Ubobo	24° 27'	151° 11'	200	tree to 18
Woondum SF	26° 15'	152° 48'	380	tree to 30
South Maroochy R	26° 34'	152° 48'	90	tree to 25
Eumundi	26° 30'	152° 59'	40	tree to 25
Noosa NP	26° 23'	153° 06'	60	tree to 25

SF, State Forest; R, River; NP, National Park.

Source: Doran and House, 1996.

The major centres of distribution are the Sunshine Coast hinterland and the Mackay - Cumberland Islands - Conway Range region, where rainfall exceeds 1050 mm annually and temperatures range from 5-28 °C, with a mean annual temperature of 18 °C (D. Lea, pers. comm.).

2.1.3 Habit and ecology

B. citriodora occurs as a mallee or tree growing up to 30 m height (Brophy *et al.*, 1995; D. Lea, pers. comm.; Taylor, 1996), and has a juvenile creeper form of up to 20 m length which can develop from both seedling growth and root suckers (Figures 2.2 and 2.3). The leaves are a glossy green on the upper-side and a pale green below, 4 - 15 cm long x 1 - 5 cm wide, with abundant oil glands (Stanley and Ross, 1986). Although it can survive for long periods in low light conditions as a creeper on the forest floor, *B. citriodora* demands stronger light for development. It thrives on the rain forest edge, and in the higher light environment of creek banks. The species has the ability to respond to these higher light environments by upwards growth of shoots from the ground creeper, or from vigorous epicormic shoots on existing trees (D. Lea, pers. comm.).

Figure 2.2 *B. citriodora*

Figure 2.3 Juvenile creeper form

B. citriodora generally prefers clay loam soils of neutral or slightly acid pH(6 - 7), with a significant humus layer in the A horizon. However, the occurrence at Ubobo is on soil derived from limestone with an alkaline pH of 8.5 (D. Lea, pers. comm.).

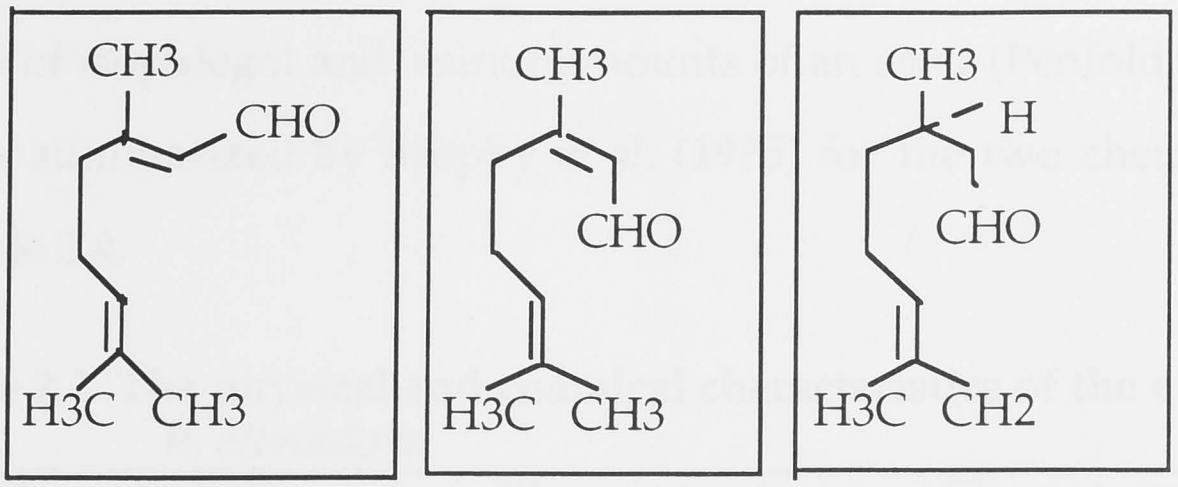
2.1.4 Reproductive and genetic characteristics

B. citriodora naturally regenerates by seed, root suckering and coppicing (Doran, 1995; D. Lea, pers. comm.). However, as its seed germination rate is very low (about 4%), the survival of the species in its natural habitat is seen to rely heavily on its vegetative reproductive capacity (D. Lea, pers. comm.), and it is this capacity which allows it to persist in the rain forest environment. Vegetative propagation is best achieved by taking tip cuttings from young seedlings (Costin, 1990). Levels and patterns of genetic variation in the species are not yet known, although preliminary studies have been initiated by CSIRO Forestry and Forest Products and the Queensland Forestry Research Institute (Doran and House, 1996; Taylor, 1996).

2.2 The essential oil of *B. citriodora*

2.2.1 The physical and chemical characteristics of the oil

The essential oil of *B. citriodora* was first reported in 1888, to consist mainly (95%) of the aliphatic aldehyde, citral $C_{10}H_{16}O$ (Figure 2.4) (Scott and Brewer, 1983). Citral is a doubly unsaturated monoterpene aldehyde, a mixture of the *cis* and *trans* isomers, namely, *trans* -Citral (C.A.), geranial, and *cis* -Citral (C.B.), neral. When heated, citral is converted to isocitral, and in the light, it cyclizes to phonocitral A (Scott and Brewer, 1983). These responses were first observed in 1923 by Penfold, in his examination of the oils of trees cultivated at Ashfield, N.S.W. (Penfold *et al.*, 1951).



1- Geranial **2- Neral** **3 - Citronellal**

Figure 2.4 Molecular formula of citral (geranial and neral) and citronellal.

Another important observation was that of Mr. J.R. Archibold in 1950, who noticed whilst distilling oil from trees near Miriam Vale, Queensland, a slight difference in odour of one of the distillates. This observation led Penfold *et al.* (1950, 1951) to examine additional single tree samples, and they identified two chemotypes. Chemotype One is distinguished by 90 - 97% citral (neral + geranial) content, whereas Chemotype Two comprises largely citronellal (63 - 68%) (Figure 2.4). Citronellal is an unsaturated monoterpene aldehyde found in both optical isomers and the iso-propylidene form. The more important form is (+)- citronellal, the chief component of citronella oils, as its tendency to cyclize is utilised in synthesis of monocyclic monoterpenes (Scott and Brewer, 1983). They also found laevo-citronellal for the first time in an Australian essential oil, with the highest optical rotation then recorded. However, no - one has since been able to locate any trees of this second chemotype until it was reidentified in this project[3]. The physical and chemical characteristics of the oil are listed in Table 2.3.

2.2.2 Chemical composition of the oil

Chemotype "1" is more common; its oil comprises, in addition to citral (90 - 95%), other monoterpenoids, principally carbonyles and alcohols. Oils of the

[3] This rediscovery of the second chemotype was a joint effort with Dr. J. C. Doran, Dr. J.J. Brophy and Mr. C. Hilliker.

apparently very rare Chemotype "2" comprise, in addition to laevo-citronellal, around 13% of isopulegol and minor amounts of an ester (Penfold *et al.*, 1951). Compounds summarized by Brophy *et al.* (1995) for the two chemotypes are listed in Table 2.4.

Table 2.3 The physical and chemical characteristics of the oil of *B. citriodora*

Characteristics	**Chemotype 1** (Type)	**Chemotype 2** (l-citronellal form)
Specific gravity (15°/15°)	0.8909 to 0.9000	0.8668 to 0.873
Refractive index, 20°	1.4859 to 1.4880	1.4527 to 1.4571
Optical rotation	Inactive to + 0.35°	- 9.8 to - 12.5°
Citral content	90% to 97%	____
Citronellal content	____	62.5% to 68%

Source: Penfold *et al.*, 1950.

2.3 Uses of the essential oil of *B. citriodora*

The extracted oil, as well as the strong-smelling foliage and abundant flowers of *B. citriodora*, can be used in aromatherapy, perfumery, food and drink flavouring, and culinary art. Small quantities of the oil have been distilled and marketed for these purposes for more than a century (Penfold *et al.*, 1951).

The therapeutic properties of *B. citriodora* oil can be attributed to the antiseptic, antiviral, antifungal, carminative, sedative and corrective properties of citral and laevo-citronellal (Atkinson and Brice, 1955; Moleyar and Narasimham, 1987; Schnaubelt, 1989; Tisserand and Balaco, 1988). The common application is to mix the oil with 45% alcohol in a ratio of 1:5, and take 3 - 4 ml three times daily. This medication is believed effective in alleviating a range of conditions: the common cold, influenza, brochitis, indigestion and other irritable gastrointestinal tract disorders, and herpes simplex (Pengelly, 1991). Laevo-citronellal from other sources is also widely used as insect repellent (Pengelly, 1991). Aldehydes, and particularly citral, have long been considered to contain anti-tumour properties (Tisserand and Balacs 1988).

Table 2.4 Compounds identified in the leaf essential oils of the chemotypes of *B. citriodora* .

Compound	Chemotype 1 (%)	Chemotype 2 (%)
1. An ethyl ester	tr	
2. C_{10} H_{14}	tr	
3. ß-pinene	0.18	
4. C_{10} H_{16} O	0.15	
5. C_{10} H_{14}	tr	
6. C_{10} H_{14}	tr	
7. E-ß-ocimene	0.01	
8. *p*-cymene	tr	
9. C_{10} H_{14} O	tr	
10. 5-methylhept-6-en-2-one	0.71	
11. a p-menthatriene*	tr	
12. *a, p*-dimethylstyrene	tr	
13. citronellal	tr	63-68
14. C_{10} H_{14} O	0.02	
15. C_{10} H_{16} O	tr	
16. C_{10} H_{16} O	0.04	
17. isopulegol		13
18. an ester		+
19. linalool	0.02	
20. C_{10} H_{16} O	0.34	
21. ß-elemene	0.09	
22. ß-caryophyllene	0.04	
23. C_{10} H_{16} O	tr	
24. neral	39.50	
25. geranial	57.78	
26. C_{10} H_{16} O	0.03	
27. nerol	0.13	
28. geraniol	0.38	
29. C_{10} H_{14} O_2	tr	
30. globulol	0.09	
31. viridiflorol	0.05	
32. spathulenol	0.35	

Note: * correct isomer not identified; + = present; tr = trace.

Source: Brophy *et al.*, 1995.

As early as the 1950s, oils from *B. citriodora* and *B. angustifolia* were identified as superior in anti-bacterial action against human pathogens, such as *Staphylococcus aureus* and *Salmonella typhii* among the 34 plants species tested in a research program (Atkinson and Brice, 1955). While citral is known to cause skin sensitisation in some people, Tisserand and Balacs (1988) found no skin sensitivity to the oil of lemon-grass, which contains up to 85% citral. Thus, there is reason to believe that the oil of *B. citriodora* could have wide therapeutic use (Pengelly, 1991), when it is more widely and economically available.

B. citriodora foliage (ground, dried leaves used as spice) and oil also has value in the flavouring and perfumery industries. Citral for these purposes was produced principally from lemon oil (5% citral), in the first instance, and then from the lemon grasses, *Cymbopogon citratus* and *Cymbopogon flexuosus* (70-85% citral). As technological development allowed the conversion of citral into ionone, an expensive and difficult synthetic perfume to produce, most lemon grass essential oil was used for that purpose (Blogg, 1920). The value of ionone promoted research into its synthesis from the essential oil of other plants, and that of *B. citriodora* was successfully converted (Blogg, 1920). Whilst the current use of *B. citriodora* oil for this purpose remains limited, the strong demand for lemon-flavoured perfumes and cosmetics indicates good prospects for its wider use.

The oil of *B. citriodora* also has much potential in food and drink flavourings, confectionery and culinary uses. The scale of this potential can be judged by the growing variety of products and recipes in which it is finding a role. A recent example is the statement in *Australian Bush Foods* (Longstaff, 1996):

> "Australian chefs have in the last ten years been combining native Australian bush food with traditional fare to create a new emerging cuisine with exciting tastes and flavours. Great combinations like lemon myrtle bavarois, gum leaf oil and lemon aspen vinaigrette

wattle creme broule or barramundi with munthari berries. Enjoy the journey of exploration of new tastes".

2.4 Market opportunities

The commercial potential for lemon myrtle looks extremely promising. Spice and leaf extracts act as both a flavour and preservative when used as food ingredients, making lemon myrtle much sought after in the world food and flavouring markets and especially in Asia, USA and Europe (B. Milgate[4] , pers. comm.). Citral which is abundant in leaves of *B. citriodora* is highly aromatic, sweetly flavoured, high in antimicrobial properties (antifungal, antiviral) and can be used as a calmative and sedative in aromatherapy. The export demand for lemon myrtle has been established from existing plantations and products, and a critical mass of high quality product is now required to supply processed Lemon Myrtle products to the world market.

Australian Native Food Resource Development Pty Ltd, an Australian company with several years experience in growing and marketing Lemon Myrtle, estimate a world demand of 3000 tonnes per annum of *B. citriodora* spice by the year 2010 (B. Milgate, pers. comm.). To meet this demand 3 million *B. citriodora* trees, or ten times the number presently under cultivation, will need to be planted over the next decade. Fresh leaf of *B. citriodora* currently trades at $12/kg, and oil at $600/l, although price of oil is expected to stabilise at around $100/l as supply increases. These prices suggest the potential of attractive returns to growers from the cultivation of *B. citriodora*.

Another interesting market profile of *B. citriodora* products can be drawn from the market information published as "Australian Native Bushfoods" by Sofcom (1996), including a list of current prices for various bush foods which use *B. citriodora* as a ingredient. Details are listed in Table 2.5.

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[4] B. Milgate, c/- John Doran, CSIRO Forestry and Forest Products.

Table 2.5 Prices for Lemon Myrtle food products

Category	Code	Ingredient name	Unit	Price
1. Robins Food	F11512	Lemon Myrtle Vinaigrette	375gm	$6.90
	F10915	Lemon Myrtle Oil	25ml	$29.95
	F10916	Lemon Myrtle Leaf Dry	100gm	$16.85
	F10937	Lemon Myrtle Leaf Ground	100gm	$19.50
2. Bush Food	F10940	Lemon Myrtle Fettucine	1kg	$24.85
Pasta	F10949	Lemon Myrtle Fettucine	250gm	$ 6.80

Source: Sofcom, March 1996.

2.5 Research on *B. citriodora*

Although *B. citriodora* was described as early as 1866, only limited research on some aspects of this species has been carried out, and little is known about its genetic variation. Apart from botanical description and investigation of cultivation methods, most of the studies carried out to date have focused on distillation and chemical composition of its oil (Blogg, 1920; Brophy *et al.*, 1995; Penfold *et al*, 1950). Selection for genetic improvement began only recently.

An attempt to identify superior sources was reported by Costin (1996), who collected several wild specimens from different geographical locations, distilled the oil, and had it analysed. The results revealed considerable individual variation in oil content and yield; the best of the selected plants contained 97.7% citral (34.6% neral and 63.1% geranial) when extracted using solvent, corresponding to 89.7% citral (40% neral and 49.7% geranial) when distilled. On the basis of this preliminary study, Costin (1996) was prepared to guarantee 3% oil content (on a fresh weight basis) when distilled from their best commercially - available selection.

Currently, a more integrated research program on *B. citriodora* is in progress at Gympie, Queensland, as a joint research project conducted by Dr John Doran of

CSIRO Forestry and Forest Products and Dr Alan House of the Queensland Forestry Research Institute. The program aims to provide growers with good quality planting stock at a reasonable price. To meet this end, the project involves assembling lemon myrtle germplasm collected from across the range of the species; gathering baseline data on the variation in commercial traits in the species; developing a selection and propagation strategy; and ultimately releasing improved genotypes for industrial use (Taylor, 1996).

Seedlings and clones are now being field tested at Beerburrum in south-east Queensland. The preliminary assessment of progeny performance showed that the average oil content was 1.5% of leaf fresh weight, compared to 1.8% for the parent trees. Individual oil content ranged from 0.9% to 2.5%, indicating that there is scope for selection for high oil concentration (Taylor, 1996). The research described in this thesis is one part of the joint CSIRO/QFRI program.

In conclusion, there is increasing demand for the products derived from *B. citriodora.* Mass vegetative propagation of cultivars selected for traits such as adaptability, growth and oil quality is likely to be the preferred means of establishing this species, because of problems with seed-based multiplication. To date little is known of the genetic base of the species, which limits development of superior genotypes. Therefore, tree breeders urgently require the genetic information for this species, so they can best use quantitative genetics as a tool for the improvement of *B. citriodora*.

CHAPTER 3. MATERIALS AND METHODS

3.1 Introduction

The evaluation of genetic variation in oil yield, biomass production and vegetative reproductive capacity of *Backhousia citriodora* reported here is based on the common approach of interpreting data from progeny and clonal trials using quantitative genetic theory and methods. As no previous genetic studies of *B. citriodora* have been carried out, some assumptions had to be made from experience with other species. Although the results reported must be interpreted in this light, they remain the best currently available for *B. citriodora*.

Data for this project were acquired from two separate trials. The first trial - Trial 1 - was established using seedlings for the assessment of provenance and family variation in oil yield, biomass production and coppicing ability. The second - Trial 2 - used cuttings from Trial 1 for the evaluation of vegetative reproductive capacity. Details of these trials are given below.

3.2. The experimental sites

Trial 1 had been established previously for progeny testing of *B. citriodora* at Beerburrum Forest Nursery in south-east Queensland. The nursery is located at the foot of the Glasshouse Mountains, latitude 26° 51' S and longitude 152° 57' E, about 66 km north of Brisbane (Figure 3.1).

The physical conditions of the nursery site are appropriate for good growth of the plants. The topography is flat and the slope less than 2°. The soil type is yellow earth with relatively high fertility and good drainage. The local climate of the area is warm and humid throughout the year, with the daytime mean temperature ranging from 22-25 °C in January and 13-16 °C in July. The annual precipitation in this area is 800-1600 mm with a median summer rainfall of 800-

1200 mm and a median winter rainfall of 400-500 mm (Department of Mapping and Surveying, 1976; Australian Surveying & Land Information Group, 1992).

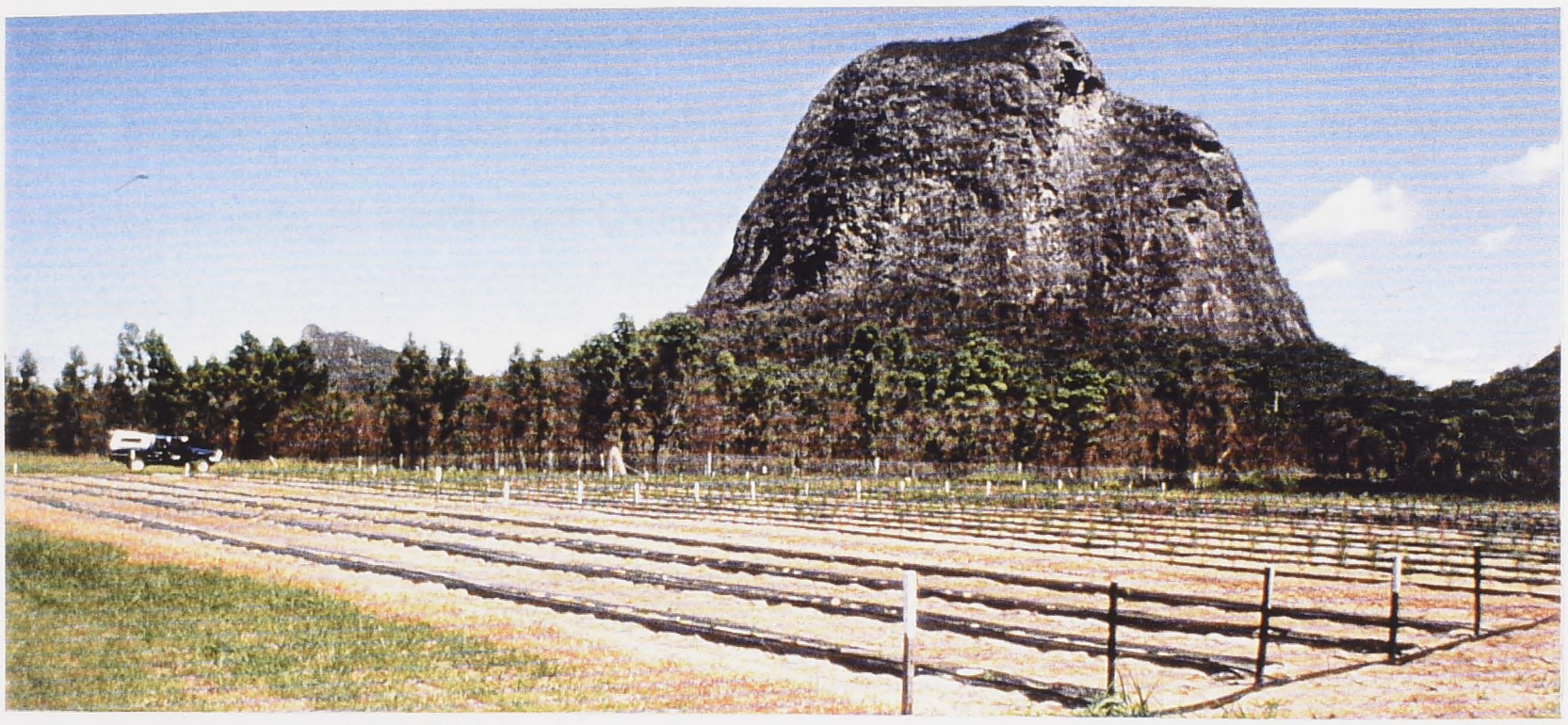

Figure 3.1 Location of experimental site for Trial 1

The second trial was carried out in the glasshouse of Queensland Forestry Research Institute at Gympie, latitude 27° 30' S and longitude 152° 45' E, about 145 km north of Brisbane (Figure 3.2). The glasshouse was well equipped with temperature and irrigation control systems which maintained the glasshouse conditions to experimental requirements.

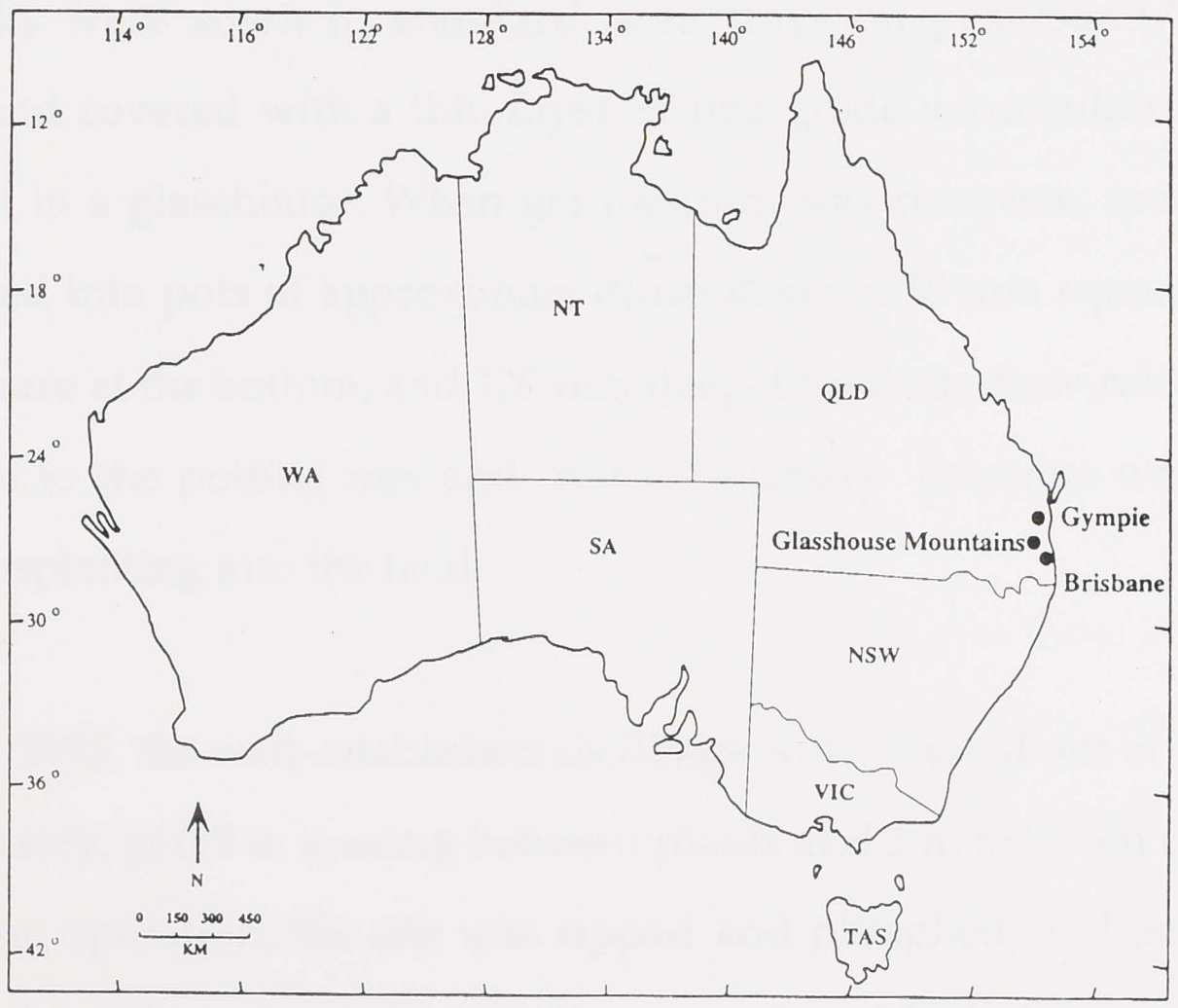

Figure 3.2 Location of experimental site for Trial 2

3.3 Genetic material and trial designs

3.3.1 Trial 1

The experimental material used in this study comprised seedlings planted in 1995, which had been hedged 4 times prior to the commencement of this study. The hedge plants were established from 16 family seedlots collected from natural stands in south-east Queensland. Details of family origins are given in Table 3.1.

Table 3.1 Provenances of the 16 families represented by hedge plants

Family code	Provenance
1009, 1010, 1011, 1012, 1028, 1029	Woondum
1013, 1014	South Maroochy River
1016, 1023, 1024, 1025, 1026, 1027	Eumundi
1030, 1031	Noosa National Park

Note: Provenance locations are mapped in Figure 2.1.

Fruits were collected between 24 and 30 April, 1994. After air drying for 2-3 days, fruits were sown in standard seed trays on peat/sand/vermiculite medium and covered with a thin layer of fine-grade vermiculite. Trays were kept moist in a glasshouse. When germination was complete, seedlings were transplanted into pots of approximate dimensions of 50 mm square at the top, 40 mm square at the bottom, and 120 mm deep. Osmocote slow-release fertiliser was added to the potting mix and normal nursery practices were followed before transplanting into the field.

In January 1995, the well-established seedlings were planted out at Beerburrum Forest Nursery, at 0.5 m spacing between plants and 2 m between rows. Before the planting operation, the site was ripped and ploughed, and Sarlon woven black weed matting was laid. Plants were placed in holes cut in matting.

Osmocote fertiliser was applied at planting. Irrigation was provided by Netafilm drip tube, with drips at intervals of about 30 cm, throughout the growing season. The stock plants were cut back to 30 cm height four times, in April, July, and November 1995 and Feburary 1996, to promote hedge development. By May 1996, the hedges were well established and ready for collection of cuttings and full assessment of oil production, biomass and coppicing ability (Figure 3.3).

Figure 3.3 Stock plants (30 April, 1996)

The design of Trial 1 comprised 4 blocks in a randomised complete block design, each comprising a single 6-tree line plot of each of the 16 families.

3.3.2 Trial 2

3.3.2.1 Cutting collection

The stock plants from which cuttings were collected were those established in Trial 1. Cuttings were collected at Beerburrum Forest Nursery on the mornings of 30 April to 3 May, 1996. It was intended that 7 cuttings should be collected from each of the 384 plants in Trial 1, yielding 2688 cuttings in total. However, some hedge plants had died or were unhealthy, and it was not possible to

collect 7 cuttings from each. Six or 7 cuttings were collected from each surviving hedge plant, yielding a total of 2029 cuttings.

Cuttings were trimmed to 8 cm in length in the field and immediately placed in plastic bags and sprinkled with water (Figure 3.4). Air was then squeezed out of the plastic bags; bags were tightly sealed, placed in cool insulated boxes and transported, at the end of the morning collection periods, to the glasshouse of the Queensland Forestry Research Institute, Gympie.

Figure 3.4 Cutting collection and treatment in the field

3.3.2.2 Setting of cuttings in the glasshouse

Setting of cuttings took place on the afternoons of 30 April to 3 May, 1996, following their collection at Beerburrum on the same morning. Prior to setting, cuttings were randomised by hand. The leaf area of each cutting was carefully reduced by 50% (Figure 3.5) and the basal end dipped in a commercial rooting powder (I.B.A.) with an auxin concentration of 0.4%. Immediately following dipping, cuttings were inserted at 1.5 cm depth into the rooting medium and firmed in position, ensuring that no leaves touched the surface of the medium, and that the medium was not waterlogged (Figure 3.5).

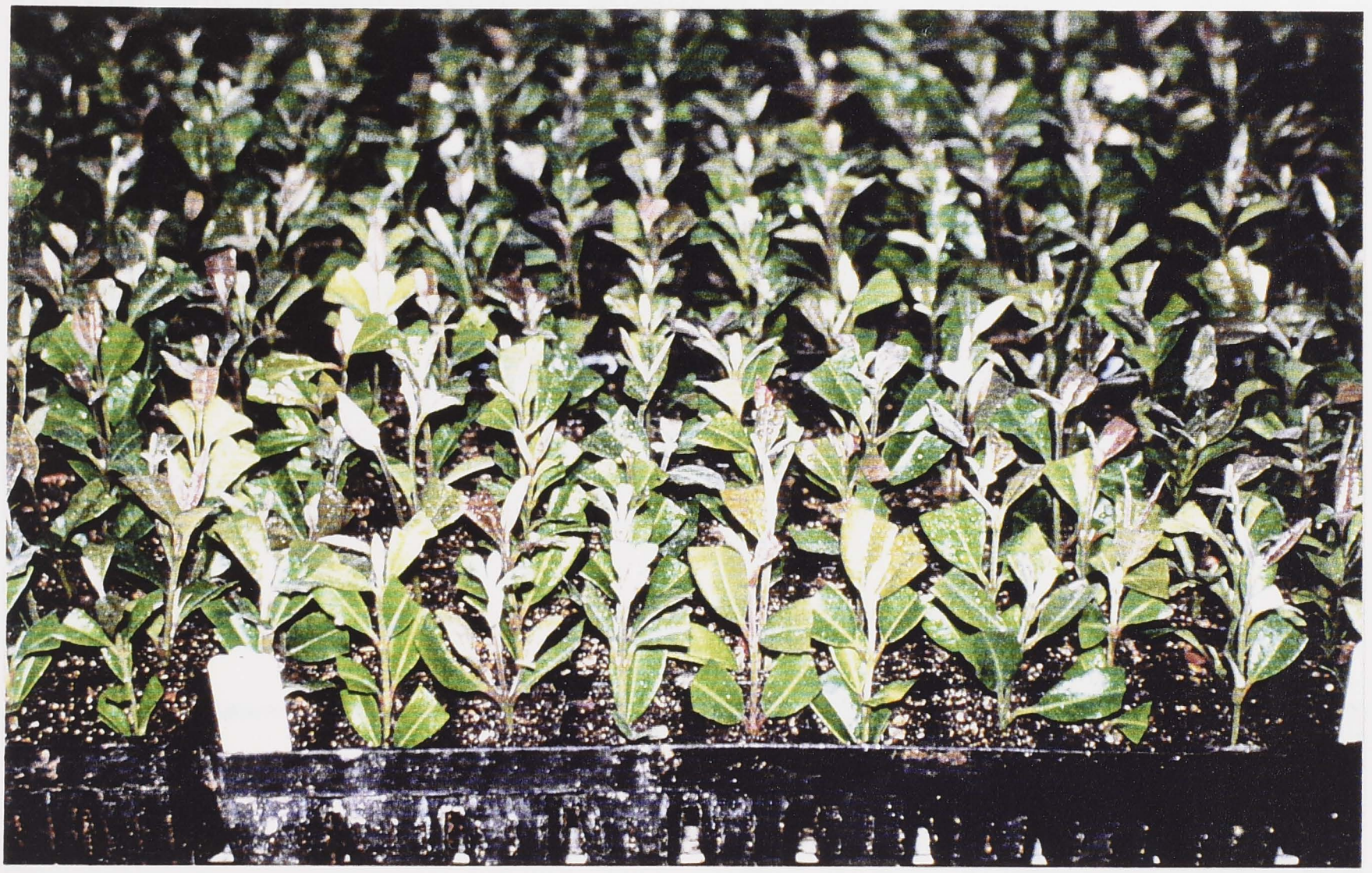

Figure 3.5 Cuttings after setting

The rooting medium consisted of peat moss and washed river sand in the proportion of 1:1, mixed with Osmocote fertiliser (1kg /m^3) which is expected to remain active for 8-9 months. Container trays, comprising 63 individual compartments, were filled with the rooting medium and left on the mist bench for one week to stabilise before the cuttings were set.

After the setting operation, cuttings were carefully tended for the duration of the experiment. Besides the careful management of environmental factors described below, cuttings were sprayed with the fungicide Benlate, at 2 g/litre, twice a week.

Trial 2 was established in a randomised complete block design of 7 blocks. Each block comprised 6 plastic trays, each of which contained 63 cells. The fifth tray in each block was only partially filled and the sixth tray in each block was left

blank. Single cuttings from each hedge plant were successively chosen at random, and set. Seven replicate groups of cuttings, corresponding to the 7 blocks in the trial, were formed in this way. The identity of each cutting was retained.

3.3.2.3 Propagation environment and tending of cuttings

The cuttings were maintained in a glasshouse at the Queensland Forestry Research Institute. A mist propagation bench was enclosed with polythene walls to minimise any micro-environmental variation in the glasshouse (Figure 3.6.).

Figure 3.6 The propagator and glasshouse environment

The misting regime was controlled by an electronic balance arm system[6] , with the rate of misting set to ensure a film of water was maintained on the leaves at all times. Ventilation was provided automatically to regulate the glasshouse temperature at between 28 and 32°C. There was no supplementary lighting, and

[6] Sage Horticultural, 121 Herald St. Cheltenham, Victoria

natural day light was reduced to 50% of full sunlight by the glass panels of the glasshouse and additional shading.

3.4 Assessment methods

3.4.1 Assessment of oil production

3.4.1.1 Methods for oil analysis

There are a number of well-established laboratory methods available for quantitative determination of essential oils (Doran, 1992). Among these methods, steam distillation combined with Gas Liquid Chromatography-Mass Spectrometry (GLC-MS) is used routinely for the quantitative and qualitative analysis of *Eucalyptus* (*eg* Doran, 1992) and *Melaleuca* (*eg* Butcher, 1994) oils. Solvent extraction-GLC is another promising method which provides a rapid, simple and sensitive approach to quantitative determination of terpenes (Ammon *et al.*, 1985). Both methods were employed by Doran (1992) and Butcher (1994) in their analysis of *E. camaldulensis* and *M. alternifolia* oils, respectively.

Results reported by Doran (1992) showed no significant difference between the two methods in quantifying four common monoterpenes in the oil of northern red gums. This, together with results reported by Ammon *et al.* (1985), confirmed the accuracy of the solvent extraction-GLC methodology for determination of terpene content, and indicated the suitability of ethanol as a solvent in which all the terpenes in *Eucalyptus* leaves are miscible; problems with oxidation and reduction are minimal, and penetration of the leaves is excellent (Doran, 1992). In order to make sure that this method works well with *B. citriodora* oil, further verification was carried out during the project. Comparisons with T-tests showed that there was no significant difference ($p < 0.05$) in citral concentration and oil yield between solvent extraction and steam distillation procedures, or in citral concentration between two-week and four-week extraction. Therefore, the solvent extraction-GLC method was adopted

with confidence in this project; however, several precautions must be taken during sample preparation and extraction.

Firstly, solvent volume must be measured accurately as it enters the calculations of the concentration of the extract and of oil yield. Therefore, solvent volume for oil extraction must be identical (set here at 50 ml) for each individual bulk sample (Butcher, 1994; Doran, 1992).

Secondly, leaf sub-samples (usually 5 g or 3 g) for oil extraction must be accurately weighed, as the weight determines the denominator of the formula for calculating oil yield (Butcher, 1994; Doran, 1992). Another factor affecting accurate calculation of oil yield is the variable amounts of internal standard added to each of the oil extracts before GLC analysis. The concentration of internal standard in the extract should ideally give a peak area for the standard of approximately the same area as the compound being quantified. As a rule-of-thumb, the addition to the 50 ml extracts of a quantity of internal standard of approximately 2% of leaf weight works well (Butcher, 1994; Doran, 1992), and was used in this study.

In addition, leaf samples must be fully immersed in the solvent during the oil extraction process and sufficient time should be given for full extraction. Normally, a period of two weeks is adequate (Doran, 1992). The total length of time for complete extraction should be decided according to experience obtained with other species, but also from pilot tests for the species of interest (Doran, 1992).

In Doran's (1992) and Butcher's (1994) studies, oil analysis was conducted on three trees selected at random from each family in each experimental plot. About 12 mature leaves were collected from each of two branches removed from the upper 2/3 of the crown (Butcher, 1994). From the bulk samples, a

subsample of leaves was used for oil extraction: either 3 g (Doran, 1992) or, sometimes, 5 or 6 g (Butcher, 1994).

In conclusion, although steam distillation has been and remains the standard method for oil extraction from plant leaf material, it is clearly impractical for a large number of samples where time and resources are limited (Butcher, 1994; Doran, 1992). The solvent extraction procedure can be considered as an alternative able to cater efficiently and accurately for a large number of samples (Butcher, 1994; Doran, 1992). In view of the large number of oil samples and limited time available, the solvent extraction method was used for oil extraction in this project. A Varian 3400 Gas Chromatography at the ANU Department of Forestry was employed for oil composition analysis.

3.4.1.2 Field collection of leaf samples

Leaf samples were collected on 4 May in 1996 from each of the 272 surviving hedge plants. Around 10 g of leaves were sampled at random from the material harvested for biomass assessment (**3.4.2.2**). Immediately after collection, sample leaves were placed in plastic bags and stored in a cool room (0 to 4 °C) for one day. They were then placed over ice in insulated boxes, and sent to Canberra within 24 hours for immediate extraction and moisture content determination.

3.4.1.3 Oil extraction

When leaf samples arrived at the laboratory in the ANU Department of Forestry, Canberra, the bulk samples were divided into two parts. One part (5 g) was used for oil extraction and chemical analysis, and the remainder was used for moisture content determination.

A 5 g subsample of leaves was accurately weighed to 0.001g and placed in 50 ml of ethanol in a plastic bottle, and left for extraction in cool and dark conditions for two weeks (**3.4.1.1**), prior to GLC analysis. To ensure full

extraction, the bottles were shaken for ten seconds each day. An internal standard of n-tetradecane (C_{14}) was then weighed to 0.001g and added by Eppendorf pipette to each sample bottle, such that each sample received a constant volume of the internal standard equal to 0.1 g, i.e. 2% of the leaf sample weight. About twelve hours later, a 1.5 ml extract from each of the sample bottle was transferred to an auto-sample vial for GLC analysis.

3.4.1.4 Technical specifications for GLC analysis

Leaf oils were analysed in ethanol extracts using a Varian 3400 gas chromatography with automatic injection and computer-based integration software (Varian Star). This method provides good sensitivity and resolution, and an accurate quantitative determination of the compounds of interest.

To meet project objectives, the gas chromatograph was operated under the following conditions: Alltech AT-35 capillary column, 30 m plus 1 m retention gap; 0.25 mm internal diameter; 0.25 μ film thickness; helium carrier gas at a flow rate of 25 cm/s (14.5 psi). The oven was programmed with the following temperature gradient:

- initial temperature was set at 80 °C which was held for 0.5 minutes;
- temperature was then increased to 100 °C at 5°C/minute;
- temperature was then raised to 210 °C at a rate of 10 °C/minute with a holding time of 5 minutes, resulting in a total run time of 20.5 minutes.

The split injector operated at 230 °C with a split rate of 70:1 and the FID detector operated at 300 °C. Each sample was run twice and rerun a third time if outside the error bounds of one percent.

3.4.1.5 Determination of oil composition and yield

(1) Oil yield

Oil yield for a fresh weight basis (O) is calculated automatically by the integration software, according to the following formula:

$$O\,(\%) = RF \times \frac{A_{(oil)}}{A_{(NTD)}} \times \frac{W_{(NTD)}}{W_{(FL)}} \times 100 \qquad (3.1)$$

where:

O is leaf oil yield on a fresh weight basis;

$A_{(oil)}$ is the peak area of oil = area (total) - area (ethanol + NTD);

NTD is the internal standard;

Area is the relevant peak area in graph;

$A_{(NTD)}$ is the peak area of internal standard;

$W_{(NTD)}$ is the weight of internal standard;

$W_{(FL)}$ is the weight of fresh leaf;

RF is the response factor of oil = $\{W_{(oil)} \times A_{(NTD)}\} / \{W_{(NTD)} \times A_{(oil)}\}$.

(2) Concentrations of neral, geranial and citral

The concentration of these components were estimated by GLC integration software. Identification of the geranial (G) and neral (N) peaks was by comparison of retention times against known standards. A small number of steam distilled samples had previously been assessed by GLC - MS analysis, and these served as standards (J.J. Brophy[5] , pers. comm.). The combined peak areas for geranial and neral were compared with total peak area to provide an estimate of citral% (C).

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[5] J.J. Brophy School of Chemistry ,University of New South Wales, Sydney.

(3) Citral yield, total oil yield and total citral yield

Citral yield was calculated on an unit leaf weight basis, while total oil yield and total citral yield were estimated on the basis of per hedge plant. The specific calculations are as follows:

$$OY = 100 \times O/(100 - M) \quad (3.2)$$

$$CY = C \times OY \quad (3.3)$$

$$TO = OY \times BL \quad (3.4)$$

$$TC = C \times TO = CY \times BL \quad (3.5)$$

where:

OY - oil yield (% of unit dry leaf weight);

O - oil yield (% of unit fresh leaf weight,);

M - leaf moisture content (% of unit fresh leaf weight);

CY - citral yield (% of unit dry leaf weight);

C - citral concentration (% of oil);

TO - total oil yield per hedge plant (g);

BL - leaf biomass per hedge plant (g);

TC - total citral yield per hedge plant (g).

3.4.1.6 Determination of moisture content

The moisture content of sample leaves was determined in conjunction with the preparation of leaf samples for oil analysis (**3.4.1.3**). After the 5 g subsamples for oil analysis was removed from each bulk leaf sample, the remainder of each fresh bulk sample was weighed again, and then placed in a paper bag and oven dried to a constant weight at 70 °C for 48 hours. After dry weight was obtained, moisture content of the leaves was calculated as:

$$\text{Leaf moisture content (\%)} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100 \quad (3.6)$$

3.4.2 Assessment of biomass production

3.4.2.1 Methods for biomass assessment

Biomass assessment is always an arduous operation which is time and labour consuming. Unfortunately, there are few shortcuts to this end for any species. There are two standard methods for biomass estimation: namely, destructive methods, in which samples are cut and weighed in the field; and non-destructive methods, in which non-destructive parameters are used to predict biomass based on regression equations (Newbould, 1967; Satoo and Madgwick, 1982). The latter method requires a well established data base developed from previous destructive sampling, which is unavailable for most species.

No matter which method is employed, the basic meaning of biomass is same, namely, the total weight of organic material in a particular sample, region, or trophic level, usually expressed as total dry weight (Wenger, 1982). The measurements taken include stems, stem bark, living branches, flowers, buds, foliage and roots of all living trees in the sample (Newbould, 1967; Vyskot, 1981). In practice, biomass is not necessarily partitioned into all these components. Depending on the purposes of particular research, leaf biomass for oil and forage, stems for timber, branches for firewood and roots for soil protection, etc., may be the only components assessed. However, the most important concerns, in practice, are the accuracy of measurements and the selection of samples. It is important that measurements be carried out with minimal delay so that post-harvest variation in moisture content can be limited to acceptable levels.

Populations have often been classified into different classes for biomass assessment, with a number - often 5 - trees sampled from each class. For example, Vyskot (1981) divided the crown of each sample tree into different layers, and collected sample branches from four aspects in each layer; others

have used different approaches. In research on *Melaleuca alternifolia*, for example, 100 hedged trees representing 22 families were fully assessed for the part higher than 30 cm above ground level (Butcher, 1994), to simulate commercial harvesting. For evaluating genetic variation, at least 24 trees should be sampled for each family (Cotterill, 1984).

3.4.2.2 Methods used in this project

To simulate commercial harvesting and use, the assessment of biomass production was based on only that part of crown 30 cm above ground, with an emphasis on leaf biomass. All hedge plants were cut back to 30 cm on 4 May 1996. Before harvesting, plant height was measured with a metre stick. Crown width was also measured with a metre stick and recorded as the average of two measurements taken at right angles to each other across the centre of the crown. The number of shoots produced by each hedge plant was counted at 30 cm above ground at the same time as biomass harvesting. In addition, the number of surviving plants per family was counted and recorded, and survival rate was calculated on a family basis as follows:

$$SV = (N_{SV} / N_t) \times 100 \qquad (3.7)$$

where:

SV - survival rate (%);

N_{SV} - number of survived trees in each family;

N_t - total number of trees in each family.

Secateurs were used for the harvesting operation. The harvested materials were immediately placed in paper bags and temporarily stored in large, insulated cool boxes. When the hedging operation was complete, all harvested materials were transported to the Queensland Forestry Research Institute, Gympie, where the fresh weight of shoots, excluding those used as cuttings in Trial 2, harvested

from each hedge plant was accurately measured. The samples were then returned to the paper bags and oven dried to a constant weight at 70 °C for 24 hours. Total weights were carefully recorded. Finally, leaves were separated from stems and weighed again, after oven-drying at 70 °C for 24 hours.

3.4.3 Assessment of vegetative reproductive capacity

As discussed in Chapter 2 (**2.1.4**), the low seed germination rate of *B. citriodora* may constrain the mass production of high quality planting stock. However, vegetative propagation by tip cuttings is seen to be an effective means of multiplication (D. Lea, pers. comm.). Since successful vegetative propagation of this species largely depends on the rooting ability of cuttings, the variation in vegetative reproductive capacity of cuttings became one of the major foci of this study.

Rooting ability refers to the ease of adventitous root initiation on cuttings and adventitious shoot initiation on root cuttings. This is typically measured by the percentage of cuttings which have established a root and shoot system and survived for some defined period of time (Land and Cunningham, 1992).

In general terms, for species that have the capacity to strike from cuttings, satisfactory rooting can be realised by creating an optimum rooting environment and by selecting appropriate propagation materials. Selection of appropriate propagation materials should include not only the selection of cutting materials from particular parts of a tree, but also recognize the importance of genetic variation in rooting ability (Doran, 1992).

3.4.3.1 Methods for evaluating rooting ability of cuttings

A number of precautions must be taken to ensure the successful assessment of rooting ability. These considerations are principally the selection of appropriate

materials, accounting for seasonal effects, control of the experimental environment, and the evaluation criteria.

In terms of the selection of material, much research has been focused on the effects of cutting origin. For example, cuttings collected from the top shoots of small *Triplochiton scleroxylon* (K. Schum.) stockplants (0.5 m) showed higher rooting percentages than those of cuttings taken from the lower parts; conversely, cuttings taken from the upper shoots of larger plants (1.5 m) had a lower root-striking percentage than those taken from the basal shaded shoots (Leakey *et al.*, 1992). A general trend deduced from research on the tropical species *Lovoa trichilioides* (Harms) was that rooting percentages of cuttings from basal shoots were highest, even in small stockplants (Leakey *et al.*, 1992). In contrast, results produced from research on *Balanites aegyptiaca* (L.) Del. confirmed that cuttings from a distal source had better rooting success and rooting quality than those from the medial and basal source (Mbah and Retallick, 1992).

Post-severance treatment and propagation environment are also key technical elements of rooting success. The following principles were developed from the reports of studies (Leakey *et al.*, 1992) on a range of tropical species. As a starting point, cuttings should be 5 - 10 cm in length, with the leaf area reduced by 50%; and auxin (0.2 - 0.4% I.B.A. in a solution of alcohol, or in the form of a commercial rooting powder), should be applied. Cuttings should be inserted to a depth of 1.5 - 2.5 cm in the rooting medium (Leakey *et al.*, 1992). The rooting medium should consist of an inert, well-aerated moisture-retaining substrate, such as mixtures of fine gravel, sand and rotted sawdust (Leakey *et al.*, 1992), or mixtures of peat moss and perlite (Mbah and Retallick, 1992). Generally, temperature optima for tropical species range from 20 - 38°C. An optimum bed temperature of 29-31°C was identified for *T. scleroxylon* at an air temperature of 25 - 30°C (Leakey *et al.*, 1992). For

B. aegyptiaca, optimum temperature of the rooting medium ranged from 31 to 37°C during the day with a minimum night temperature of 22°C in the mist unit (Mbah and Retallick, 1992). In addition, the propagator should be shaded to about 25% of full sunlight in order to keep it as cool and humid as possible and yet provide enough light for the promotion of physiological activity (Leakey *et al.*, 1992).

The criteria employed in evaluating rooting ability are crucial for assessment of genetic variation in the vegetative reproductive capacity of a species. Generally, there are two kinds of approach: namely, partial assessment, which is based on the incidence of rooting only; and full assessment, in which both incidence of rooting and root quality are taken into consideration. For the latter, a score on a point scale is employed in assessing rooting quality. The scoring system employed may vary between experiments, depending on the objectives and the time and labour available. For example, a six point scale was used by Mbah and Retallick (1992) in their research on vegetative propagation of *B. aegyptiaca*. The full assessment approach is the preferable practice for rooting assessment, as it gives a better indication of rooting ability and potential deployment.

A minimum of 40 - 60 cuttings per treatment or clone/treatment interaction is required for rooting assessment (Leakey *et al.*, 1992). Cuttings should be blocked in the propagator according to the spatial variation in the propagation environment (Newton and Jones, 1993). Treatments should be replicated across all blocks and randomised within a block, following standard practice (Leakey *et al.*, 1992).

3.4.3.2 Methods used in this project

Rooting was assessed between 15 - 18 July 1996, eight weeks after insertion of the cuttings. To better understand the vegetative reproductive capacity of this species, all cuttings were fully assessed. Traits assessed were:

- rooting success;
- rooting quality;
- plant health.

Scoring systems were developed to describe plant health and rooting quality. There were two rooting success measures, namely RS-O and RS-R. The same raw data produced from Trial 2 were used for both assessments, but were incorporated separately into the data sets for Trial 1 and Trial 2. RS-O is a measure of rooting success on an ortet mean basis, and RS-R the corresponding measure on an individual ramet basis. The details are described below:

$$RS\text{-}O = N_r / N_C \qquad (3.8)$$

where:

RS-O - rooting success;

N_r - number of rooted cuttings (cuttings with a rooting score >1);

N_C - total number of the cuttings collected from each ortet.

Rooting success (RS-R) was scored as 1 if the cutting had a rooting score > 1, and 0 if it did not. Plant health and rooting quality were assessed on the following scales:

Plant health scores (Figure 3.7)

1 cutting dead, all leaves fallen;

2 cutting dry, leaves and stem wilting and apex curled;

3 leaf tips twisted and wilting;

4 plant growing vigorously and of average size;

5 growth and size superior.

Rooting quality scores (Figure 3.8)

1 cutting without roots, very easily uprooted;

2 at least one small root formed (about 1 - 3 mm in length), easily uprooted;

3 fair rooting (2 - 8 cm in length), difficult to uproot;

4 excellent rooting, vigorous growth (4 - 9 cm in length), very difficult to uproot.

Figure 3.7 Plants with different health scores

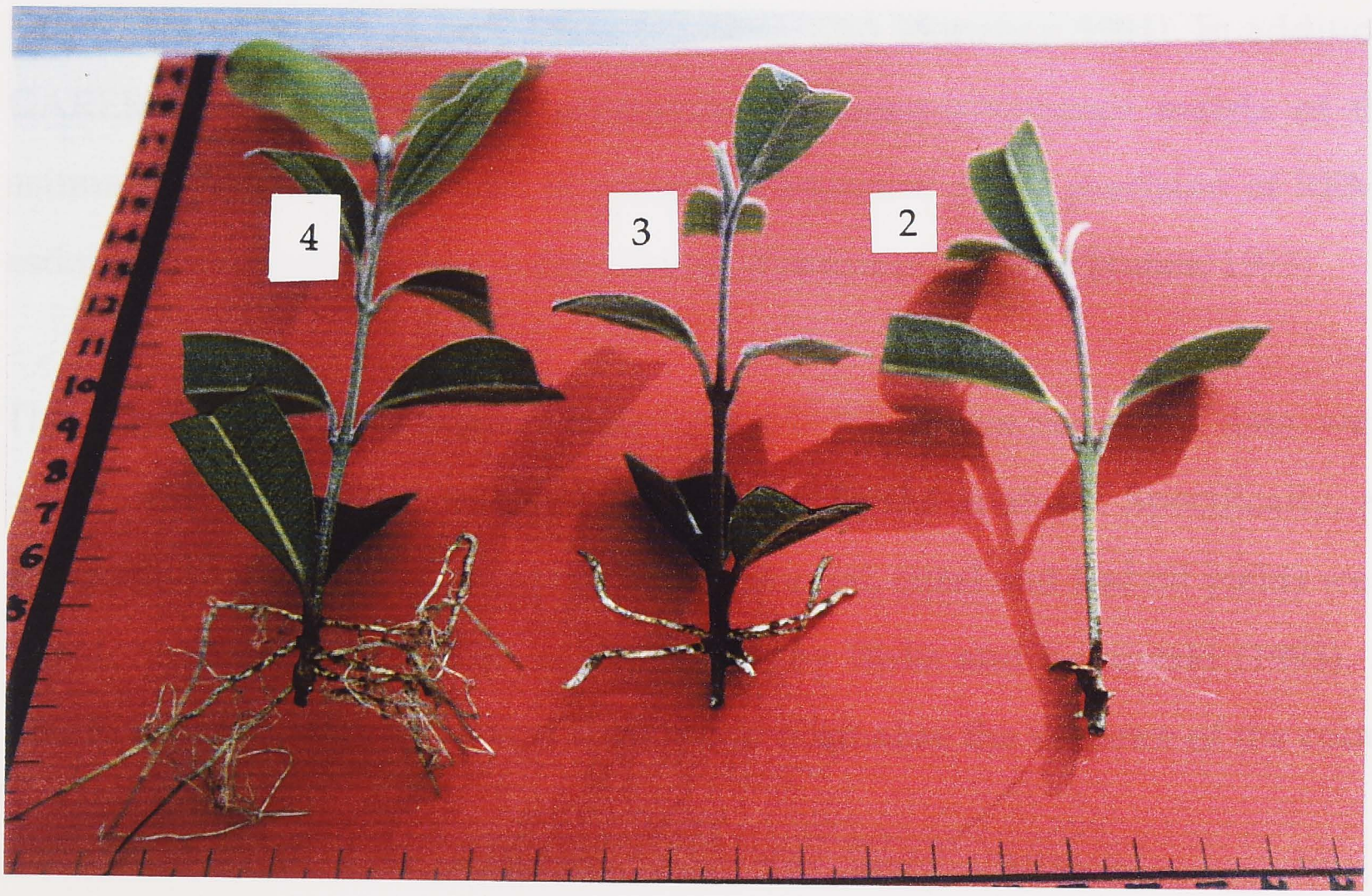

Figure 3.8 Plants with different rooting scores

To guarantee the quality of assessment of rooting success, a preliminary investigation on the ease of uprooting was carried out on 30 samples (10 for each of the scoring scales 2, 3, and 4). The data based on the assessment of number of roots, length of the longest root and plant height showed a good correlation (r = 0.72 - 0.89) between the ease of uprooting assessed qualitatively, by grasping the plant and judging its resistance to uprooting, and the score assessed after uprooting. Thus, the qualitative method was used for all plants in categories 3 and 4 in order to minimise the damage caused by the uprooting process. The remaining plants in categories 1 and 2 were assessed after uprooting, as they were easy to uproot.

3.5 Data analysis

Data were analysed, in collaboration with Dr M.J. Dieters[7], using the SAS package (SAS Institute, 1988) and a Restricted Maximum Likelihood (GAREML) program (Dieters *et al.*, 1995). The GAREML procedure offers advantages over ANOVA-based estimators as its estimates have some useful large-sample statistical properties (Swallow and Monahan 1984). In addition, GAREML also provides the asymptotic variance-covariance matrix of the estimates which can be used to provide a measure of the precision of REML estimates, even when sample size is small (Dieters *et al.*, 1995; Huber, 1993).

The traits analysed are those reported in Table 3.2. Where necessary, data were transformed to satisfy assumptions inherent in the analysis. Specifically, all compositional data (percentage of neral, geranial, citral and leaf oil yield) were transformed to natural logs, as described by Birks and Kanowski (1993). A square root transformation was required to normalise the variance of the count of shoot number.

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[7] Dr M.J. Dieters, Principal Geneticist, Queensland Forestry Research Institute, MS 483, Gympie, QLD 4570, Australia.

The linear models used in the analysis were, for Trial 1:

$$Y_{ijk} = \mu + B_i + F_j + BF_{ij} + E_{ijk} \qquad (3.9)$$

where:

Y_{ijk} - the observation on the kth tree in the jth family and in the ith block;

μ - the overall mean of the population;

B_i - the random effect of the ith block [$E(B_i) = 0$, $Var(B_i) = V_b$];

F_j - the random effect of the jth family [$E(F_j) = 0$, $Var(F_j) = V_f$];

BF_{ij} - the interaction between ith block and jth family [$E(BF_{ij}) = 0$, $Var(BF_{ij}) = V_{bf}$];

Eijk - a random error associated with the kth tree in the jth family and in the ith block [$E(E_{ijk}) = 0$, $Var(E_{ijk}) = V_e$].

Table 3.2 Traits analysed with GAREML

Trial	Trait	Unit	Transformation
1	neral	%	natural log
	geranial	%	natural log
	citral	%	natural log
	leaf oil yield	%	natural log
	total leaf oil	g	
	total citral yield	g	
	leaf biomass	g	
	hedge height	cm	
	crown width	cm	
	shoot number	count	$\sqrt{shoots+0.375}$
	survival rate	%	
	rooting success (RS-O)	%	
2	rooting success (RS-R)	%	
	rooting score	1 - 4	
	plant health	1 - 5	

and for Trial 2:

$$Y_{ijk} = \mu + B_i + F_j + BF_{ij} + C_{jk} + E_{ijk} \qquad (3.10)$$

where:

Y_{ijk} - the observation on the kth tree in the ith block and in the jth family;

μ - population mean considered as a fixed effect;

B_i - the random effect of the ith block [$E(B_i) = 0$, $Var(B_i) = V_b$];

F_j - the random effect of the jth family [$E(_{Fj}) = 0$, $Var(_{Fj}) = V_f$] ;

BF_{ij} - the interaction between the ith block and the jth family [$E(BF_{ij}) = 0$, $Var(BF_{ij}) = V_{bf}$];

C_{jk} - the random effect of clone of kth tree within the jth family [$E(C_{jk}) = 0$, $Var(C_{jk}) = V_c$];

E_{ijk} - a random error associated with the kth tree in the ith block and in the jth family [$E(E_{ijk}) = 0$, $Var(E_{ijk}) = V_e$].

3.5.1 Parameter estimates

All parameter estimates were based on the assumption that progeny within the open-pollinated families had a coefficient of relationship of 1/2.5, as is commonly assumed for *Eucalyptus* with a mixed mating system (Williams and Matheson, 1994). In the absence of any information on the reproductive biology of *B. citriodora*, this assumption, based on information for the most widely-studied genera in the Myrtaceae, seems appropriate. However, it is an arbitrary choice, and must be considered only as a first approximation until information on the reproductive biology of *B. citriodora* becomes available. As Dieters *et al.* (1995) observe, estimates obtained from a single site, such as those reported here, are likely to be biased upwards because of the confounding effects of genotype by environment interaction. The estimates also assume that dominance effects are absent, and will be inflated if they are not. Similarly, the estimation of genetic parameters does not account for provenance effects, which may be substantial. The omission of provenance as a term in the analytical

models is due to the small number of families represented, which will lead to very imprecise estimates. However, the parameters estimated here are likely to be inflated over the true values.

3.5.1.1 GCA and breeding value estimates

GCA (General Combining Ability) estimates are generated by GAREML on the assumption that the coefficient of relationship between half-sibs is 1/4. On this basis, breeding values are twice (ie, $\sqrt{4}$) the GCA estimates; here, the appropriate coefficient is $\sqrt{2.5}$. Breeding values were thus estimated as:

$$\text{Breeding value} = \sqrt{2.5} \times \text{GCA} \qquad (3.11)$$

3.5.1.2 Estimates of variance components and heritabilities

Variance components were estimated directly by GAREML, and heritabilities estimated according to the following expressions. Variation between blocks was excluded from the calculation of phenotypic variation, ie, estimates were made on a block-adjusted basis (Cotterill and Dean, 1990).

- for traits assessed on seeding material (Trial 1):

$$h^2 = \frac{V_A}{V_p} \approx \frac{2.5\,V_f}{V_e + V_{bf} + V_f} \qquad (3.12)$$

where:

V_A - additive genetic variance;

V_p - block-adjusted phenotypic variance;

V_e, V_{bf} and V_f are as described for Equation (3.9).

McGuirk (1989) and Dieters (1996) have reviewed literature relevant to the estimate of heritability of binomial data, such as that for survival (SV) and

rooting success (RS-O and RS-R) here. As Dieters (1996) commented: "these data are assumed to describe threshold traits, with an underlying normal distribution of genetic and environmental values, which are not expressed until a certain threshold value is reached on the underlying normal scale (Dempster *et al.*, 1950). This implies a simple linear relationship between heritability on the normal scale and heritability estimated on the observed binomial scale". This relationship can be expressed as follows (Dieters 1996):

$$h^2_{0/1} = \frac{h^2_n z^2}{p(1-p)} \qquad (3.13)$$

where:

$h^2_{0/1}$ - the heritability estimated on the binomial (0/1) scale;

h^2_n - the heritability on the underlying normal scale;

z - the height of the ordinate of the normal distribution at the threshold point which corresponds to the observed incidence of the trait(p).

p - the observed incidence of the trait.

• for traits assessed on clonal material (Trial 2):

Heritabilities of traits assessed on cuttings were estimated on a broad-sense block-adjusted basis.

$$H^2 = \frac{V_f + V_c}{V_f + V_{fb} + V_c + V_e} \qquad (3.14)$$

where these terms are described for Equation (**3.10**).

The standard errors of variance components and heritabilities were estimated on the basis of a Taylor series approximation (Dieters *et al.*, 1995).

3.5.1.3 Estimates of genetic correlations

The genetic correlation between any pair of traits is, according to Williams and Matheson (1994), normally estimated as :

$$r_g = \frac{Cov_f\,(x,y)}{[V_f\,(x)\,.\,V_f\,(y)]^{1/2}}; \qquad (3.15)$$

Where:

r_g - genetic correlation;

$Cov_f\,(x,y)$ - covariance of the two traits at family level;

$V_f\,(x)$ - family - level variance components of trait (x);

$V_f\,(y)$ - family - level variance components of trait (y).

However, many estimates made on this basis for this data set were well outside the bounds of feasibility (-1 to +1), presumably reflecting the small population size and consequent difficulties in accurate estimation of variance and covariance components. As a result, an alternative procedure, based on the calculation of simple correlations between breeding value predictions, was used here. Although based on predicted breeding values, this estimate is consistent with the concept of genetic correlation as the correlation of breeding values (Falconer, 1981), and returned more feasible estimates of genetic correlations in this case. However, as each trial was established at only one site, it will confound environmental and genetic correlations, and thus inflate the estimates.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Population means

Phenotypic parameters for traits assessed in this project are summarised in Table 4.1.

Table 4.1 Population means, associated standard deviations, coefficients of variation, and minima and maxima

Trait	Abbrev.	Unit	N	Mean	s.d.	CV%	Min.	Max.
neral	N	% of oil	278	33.9	3.68	11	0.29	36.3
geranial	G	% of oil	278	51.0	5.41	11	0.57	53.6
citral	C	% of oil	278	85.0	8.94	11	0.86	88.5
citral yield	CY	% of leaf	278	3.85	1.60	42	1.20	7.90
oil yield	OY	% of leaf	278	4.53	1.86	41	1.41	9.29
total oil yield	TO	g	278	1.72	1.51	88	0	8.1
total citral yield	TC	g	278	1.46	1.29	88	0	6.88
leaf biomass/plant	BL	g	302	32.4	24.9	77	0	144
hedge height	HH	cm	302	72.4	22.8	32	11.0	114
crown width	CW	cm	302	67.5	18.1	27	19.0	116
number of shoots	NS	count	302	26.9	18.1	70	0	86
survival	SV	%	384	79	41	52	0	100
rooting success-ortet	RS-O	%	291	63	25	40	0	100
rooting success-ramet	RS-R	%	2029	63	48	76	0	100
rooting score	R	1-4	2029	2.61	1.36	65	1	4
plant health	H	1-5	2029	3.62	0.84	23	1	5

The mean oil yield of this population was 4.5 % on a dry weight basis, equivalent to 2.6% on a fresh leaf basis. This is comparable with previous results reported for *B. citriodora* from natural stands by Doran and House (1996) and Costin (1990), who respectively found oil yields of 1.5 - 1.8% and 3% when distilled on a fresh weight basis. The large range and coefficient of variation indicate ample scope for improvement in oil yield through selection.

At an average of 85%, citral concentration was slightly less than that reported previously for *B. citriodora*, of >90% (Doran and House, 1996; Southwell, 1996). Similarly, average neral and geranial concentrations, at 34% and 51% respectively, were slightly lower than those reported previously, of 40% and 58% (Brophy *et al.*, 1995). These differences may be due in part to different

methodologies used for extraction and analysis. They may also be due to differences in the developmental stage of the plants, but there is no information available to verify this hypothesis. While, the extreme values of neral, geranial and citral suggest a wide range of variation in these traits among individuals, the coefficients of variation (CV=11%) are more modest. The extreme values for these traits represent only three plants, which led to the rediscovery in this project of the previously reported Chemotype 2.

Standard deviations associated with growth traits were high, ranging from 27% to 77% of the corresponding means, indicating substantial individual variation within the population. At 79%, survival rate was reasonable for this species, suggesting good potential for deployment.

Measures of rooting success averaged 63%, somewhat less than the 84% achieved in a preliminary project conducted by Queensland Forestry Research Institute (S. Walker[8], pers. comm.). These contrasting results may be attributable to technical and climatic factors at collection and in the setting and tending processes. However, they also suggest great potential for improvement through improved cultivation techniques.

4.2 Variance components

Table 4.2 summaries the magnitude of variance components in absolute and relative terms. For hedge plants, the overall trend for most of the traits, with a few exceptions, was for the proportion of variance to increase in the order of V_b, V_{fb}, V_f and V_e . The within-plot error (V_e) was the most important source of variation for most of the traits, accounting for 46-87%, while the variation due to block effects contributed least to total variability for all traits, comprising 0-9% of the total. Families (V_f) were the second most important

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[8] Mr. S. Walker, Propagation Scientist, Queensland Forestry Research Institute, MS 483, Gympie, QLD 4570, Australia.

Table 4.2. Variance components in absolute and relative terms

Source	Absolute variance values						Percentage of total variance				
	V_b	V_f	V_c	V_{fb}	V_e	V_p	V_b	V_f	V_c	V_{fb}	V_e
Trait											
Trial 1 (Hedge plants)											
Oil											
N*	0.004	0		0.025	0.189	0.218	1.9	0		11	87
G*	0.004	0.004		0.027	0.180	0.216	2.0	2.0		12	83
C*	0.004	0		0.027	0.183	0.214	2.0	0		12	86
CY*	0.005	0.174		0.024	0.179	0.383	1.4	46		6.2	47
OY*	0	0.130		0.013	0.035	0.177	0	73		7.1	19
TC	0.033	0.816		0.008	0.819	1.677	2.0	49		0.5	49
TO	0.064	1.088		0.013	1.130	2.294	2.8	47		0.6	49
Growth											
BL	46.70	142.3		2.561	406.2	597.9	7.8	24		0.4	68
HH	26.55	171.2		43.98	290.9	532.7	5.0	32		8.3	55
CW	4.800	22.93		31.35	273.5	332.5	1.4	7.0		9.4	82
NS*	0.270	1.022		0.366	2.623	4.280	6.3	24		8.5	61
Repro.											
SV	0	0.010		0.018	0.142	0.169	0	5.6		11	84
RS-O	0	0.017		0.003	0.045	0.065	0	26		4.6	70
Trial 2 (Cuttings)											
RS-R	0.009	0.017	0.021	0.004	0.186	0.237	3.9	7.1	8.9	1.8	78
R	0.113	0.161	0.2247	0.044	1.334	1.876	6.0	8.6	12	2.3	71
H	0.020	0.032	0.0578	0.011	0.595	0.715	2.8	4.4	8.1	1.5	83

Note: * Traits transformed as described in Table 3.2.

source of variation, constituting 23-75% of the total, and the interaction between family and block (V_{fb}) contributed 0.5-11% to the total phenotypic variation. A similar pattern in the relative magnitude of variance components was evident for cuttings, with V_c comparable in magnitude to V_f. In both cases, it is apparent that the variation associated with blocks across the experimental site was very small, and that the total phenotypic variation was mainly attributable to the random individual variation within families, to variation between clonal ramets in the case of cuttings, and to variation between families. The relative magnitudes of variance components are represented in Figure 4.1.

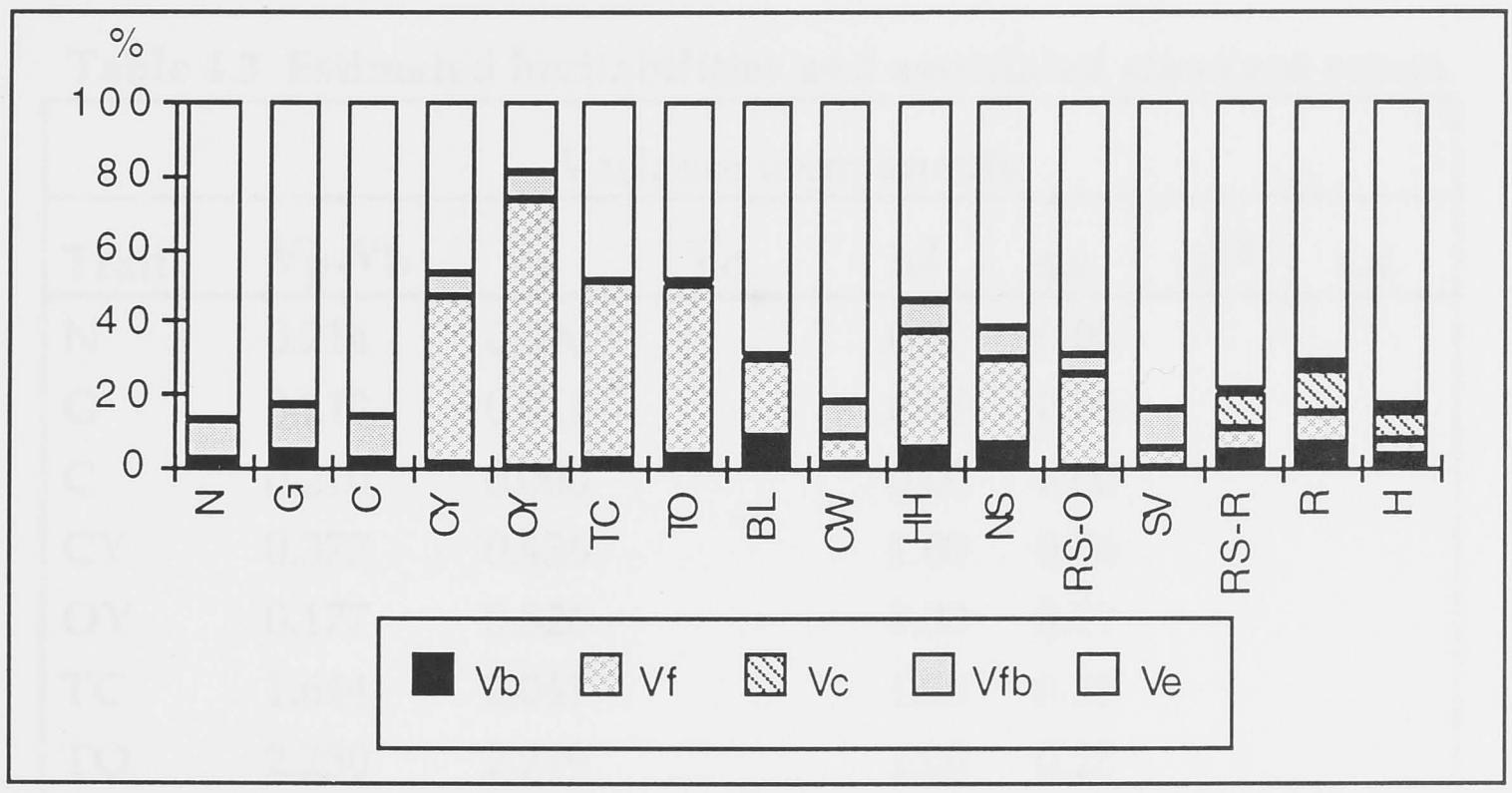

Figure 4.1 Relative magnitude of variance components

There were some exceptions to this general pattern. For the concentrations of neral, geranial and citral, there was negligible between-family variation. In contrast, between family variance was the greatest source of variation, at around 75%, for oil yield. In the cases of citral yield, total oil and total citral, the family component of variance was equivalent in magnitude to the error variance, at between 45 and 49% (Figure 4.1).

4.3 Heritability estimates

Block effects were considered fixed and were excluded from calculation of the total phenotypic variation in the estimation of genetic parameters (Cotterill and Dean, 1990). Relevant variance components, heritabilities and associated standard errors are reported in Table 4.3.

Generally, the heritability estimates are high for most of the traits assessed in Trial 1 (hedge plants), with narrow sense estimates ranging from 0 to 1, and moderate for those traits evaluated in Trial 2 (cuttings), with broad sense estimates varying between 0.2 and 0.4.

Table 4.3 Estimated heritabilities and associated standard errors

	Variance components						
Trait	V_p-V_b	V_a	V_c	h^2	s.e.	H^2	s.e.
N	0.214	0.000		0.00	0.00		
G	0.212	0.011		0.05	0.09		
C	0.210	0.000		0.00	0.00		
CY	0.377	0.436		1.00	0.26		
OY	0.177	0.326		1.00	0.19		
TC	1.644	2.041		1.00	0.25		
TO	2.230	2.719		1.00	0.25		
BL	551.1	355.9		0.65	0.21		
HH	506.1	428.1		0.85	0.25		
CW	327.7	57.33		0.17	0.13		
NS	4.010	2.555		0.64	0.22		
SV*	0.169	0.024		0.28	0.11		
RS-O*	0.065	0.042		1.00	0.22		
RS-R*	0.228	0.042	0.184			0.27	0.84
R	1.763	0.402	0.228			0.36	0.89
H	0.695	0.079	0.113			0.20	0.74

Note: * Estimates were transformed to the normal scale (Equation 3.13).

Heritability estimates were greater than one for citral yield (CY), oil yield (OY), total citral (TC), total oil (TO) and rooting success (RS-O). These estimates were

reported as unity. These inflated estimates may be due to the relatively limited number of families represented in the population, and the arbitrary use of 1/2.5 as the coefficient of relationship. In the case of RS-O, which is based on the mean values of RS-R, estimates will also reflect variation in the number of ramets per ortet and the consequent accuracy of estimates of the mean. Although these heritabilities were overestimated, they nevertheless suggest the strength of genetic control (Figure 4.2).

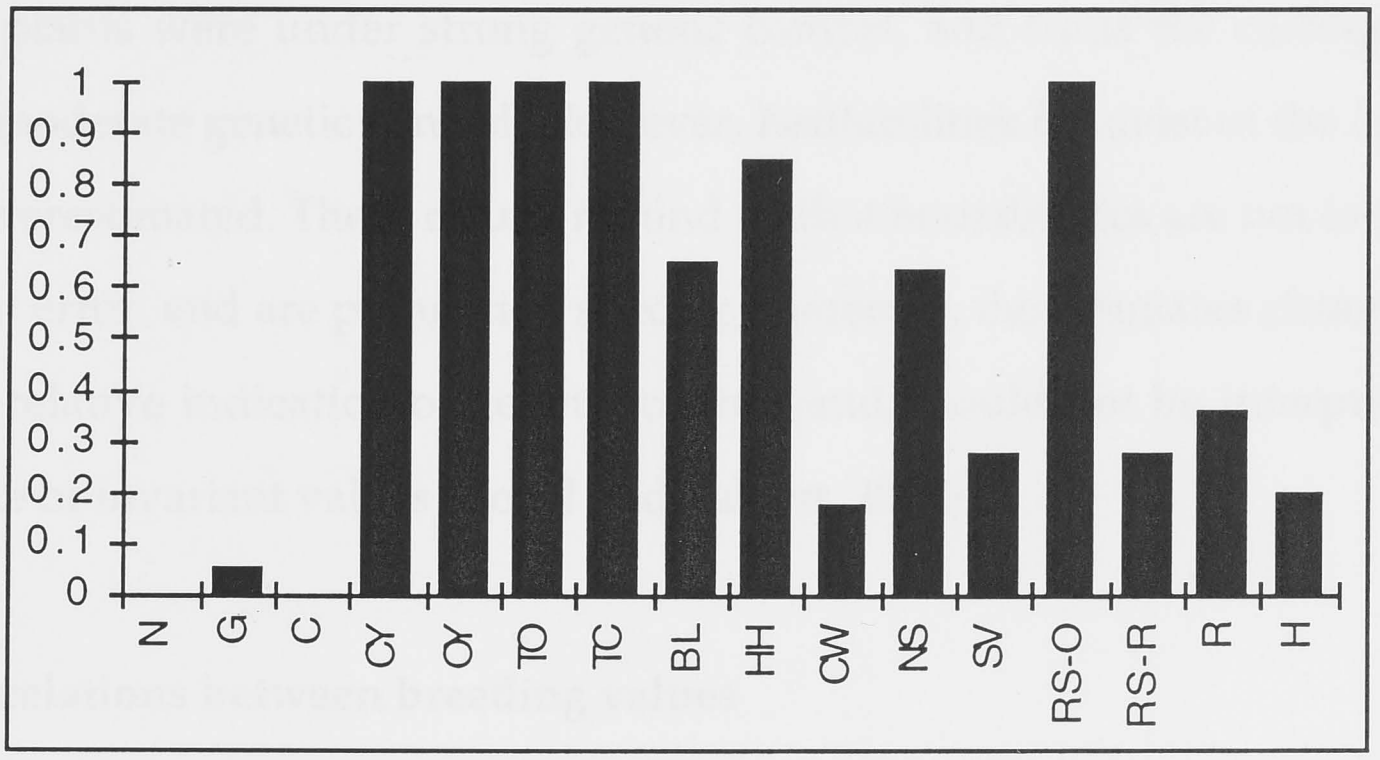

Figure 4.2 Estimates of heritability for all traits

Heritability estimates for neral, geranial and citral were very low, namely 0, 0.05 and 0 respectively, a consequence of the lack of between family variation of these traits. In contrast, the heritability estimate for citral yield exceeded one. The explanation for these contrasting results is that the measurements of neral, geranial and citral were the percentage components of the oil, and independent of oil yield. However, citral yield is calculated relative to leaf weight, and varies with oil yield.

With two exceptions, the values of heritabilities for growth and propagation traits in Trial 1 were high and comparable, ranging from 0.6 to 0.8, indicating

good potential for improvement. Crown width and survival rate had lesser, but still moderate, heritability estimates.

Broad sense heritability estimates for cuttings were moderate, at 0.2 for cutting health, 0.27 for rooting success and 0.36 for rooting score. Standard errors associated with these estimates were high.

To conclude, most of the growth and propagation characteristics assessed for hedge plants were under strong genetic control, and traits for cuttings were under moderate genetic control. However, heritabilities for most of the oil traits were overestimated. These results remind us that heritabilities are not estimated without error, and are population specific; therefore, the estimates obtained are only a relative indication of genetic control and should not be interpreted as absolute or invariant values (Zobel and Talbert, 1984).

4.4 Correlations between breeding values

Correlations between predicted breeding values are presented in Table 4.4. The estimated correlations between most of the traits assessed in each of the trials were large and positive, varying between 0.5 and 0.99. It is apparent that these characters are strongly associated and selection for one is likely to affect the other favourably. However, there were several important exceptions to this generalization. For example, rooting success on a hedge plant mean basis (RS-O) was negatively correlated with all traits other than geranial, with correlations varying between -0.1 and -0.57. Survival (SV) had very weak correlations (both positive and negative) with all traits, ranging from about -0.2 to about 0.1, suggesting little association with other traits. Correlations associated with neral and citral could not be estimated because of the lack of between-family variation in these traits. The correlations associated with geranial varied greatly with traits, ranging from about -0.2 to 0.72. Correlations between traits assessed on cutting plants (Trial 2) were all greater than 0.95.

Correlations between important subsets of traits are represented in Figure 4.3, and discussed below.

Table 4.4 Correlations between predicted breeding values for all traits

Trait	N	G	C	CY	OY	TC	TO	BL	CW	HH	NS	RS-O	SV
N	.												
G	-	.											
C	-	-	.										
CY	-	0.72	-	.									
OY	-	0.54	-	0.97	.								
TC	-	0.40	-	0.86	0.91	.							
TO	-	0.35	-	0.84	0.90	0.99	.						
BL	-	0.15	-	0.58	0.67	0.90	0.91	.					
CW	-	-0.03	-	0.38	0.47	0.68	0.69	0.78	.				
HH	-	0.06	-	0.56	0.67	0.87	0.88	0.95	0.78	.			
NS	-	0.21	-	0.65	0.72	0.88	0.88	0.91	0.69	0.88	.		
RS-O	-	0.34	-	-0.1	-0.24	-0.42	-0.45	-0.57	-0.56	-0.56	-0.43	.	
SV	-	-0.19	-	-0.05	0.04	0.05	0.06	-0.06	-0.15	0.07	-0.18	0.021	.

	RS-R	R	H
RS-R	.		
R	0.99	.	
H	0.96	0.96	.

Note: '-' represents correlations that cannot be estimated.

Citral yield and oil yield had moderate correlations with leaf biomass and coppicing ability (NS), and total citral and total oil were highly correlated with these traits. These results illustrate that total citral and total oil depend not only on oil yield, but also on leaf biomass and coppicing ability. Rooting success was negatively correlated with oil traits other than geranial, implying that breeding for increasing oil yield may decrease rooting success and hence adversely affect deployment potential using cuttings. Survival of seedling stock had a negligible association with oil traits, but is important for multiplication .

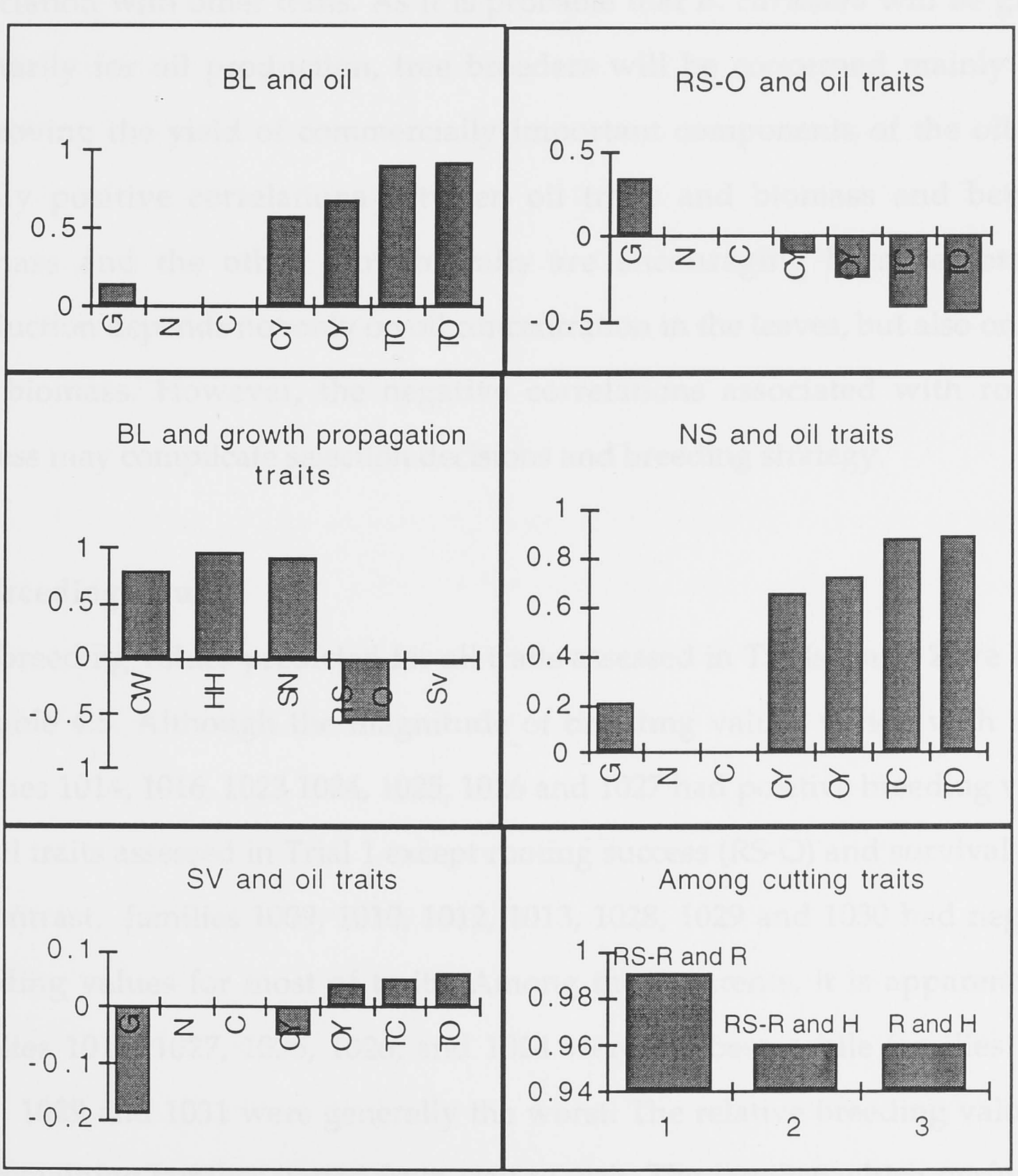

Figure 4.3 Correlations between sets of traits

Growth traits were highly correlated among themselves, with estimates between 0.69 and 0.96 (Table 4.4). Leaf biomass was strongly associated with hedge height, coppicing ability and crown width. Rooting success and survival had similar associations with leaf biomass as they did with oil traits.

In summary, oil traits - except neral, geranial and citral - were highly correlated with leaf biomass, and growth traits showed a strong correlation with each other, indicating that the selection of any one trait will affect the others favourably. The very weak correlations associated with survival suggested little association with other traits. As it is probable that *B. citriodora* will be grown primarily for oil production, tree breeders will be concerned mainly with improving the yield of commercially important components of the oil. The highly positive correlations between oil traits and biomass and between biomass and the other growth traits are encouraging because total oil production depends not only on oil concentration in the leaves, but also on total leaf biomass. However, the negative correlations associated with rooting success may complicate selection decisions and breeding strategy.

4.5 Breeding values

The breeding values predicted for all traits assessed in Trials 1 and 2 are listed in Table 4.5. Although the magnitude of breeding values varied with traits, families 1014, 1016, 1023 1024, 1025, 1026 and 1027 had positive breeding values for all traits assessed in Trial 1 except rooting success (RS-O) and survival (SV). In contrast, families 1009, 1010, 1012, 1013, 1028, 1029 and 1030 had negative breeding values for most of traits. Among these parents, it is apparent that families 1016, 1027, 1025, 1026, and 1024 were the best, while families 1009, 1028, 1029 and 1031 were generally the worst. The relative breeding values of the remaining families varied from trait to trait. The families which performed best as seedlings in Trial 1 were not those which performed best for the traits

assessed on cuttings inTrial 2, reflecting adverse correlations between seedling production traits and the rooting success of cuttings.

Families 1012, 1013, 1023, 1024, 1028 and 1029 had positive breeding values for all the traits assessed in Trial 2. However, families 1016, 1014, 1025, 1027 and 1031, most of which performed well in Trial 1, had negative breeding values for the traits assessed in Trial 2. The remainder of the parents had breeding values around the average. Individual family performance is illustrated for each trait in Figures 4.4 - 4.6, and summarised briefly below.

(1) Estimated breeding values for growth traits

Parental breeding value ranking for leaf biomass, hedge height and the number of shoots were generally similar, with parents 1014, 1016, 1023, 1024, 1025, 1026, 1027 performing well and parents 1009, 1010, 1012, 1013, 1028, 1029 and 1030 performing poorly. Among these, parents 1016 and 1027 were the best, and parents 1009, 1028 and 1029 the poorest.

Although the same groups of parents, in general, had either positive or negative breeding values for crown width as for other growth traits, relative rankings varied. Contrary to their performance for other growth traits, parents 1023 and 1026 had negative breeding values, and parent 1031 a positive value, for this trait.

(2) Estimated breeding values for oil traits

Families 1014, 1016, 1023, 1024, 1025, 1026, 1027 had positive breeding values for oil traits, with parents 1025 and 1016 the best for citral yield and oil yield. Parent 1016 was superior for total citral and total oil production, and parents 1024, 1025, 1026 and 1027 had moderate breeding values for these traits.

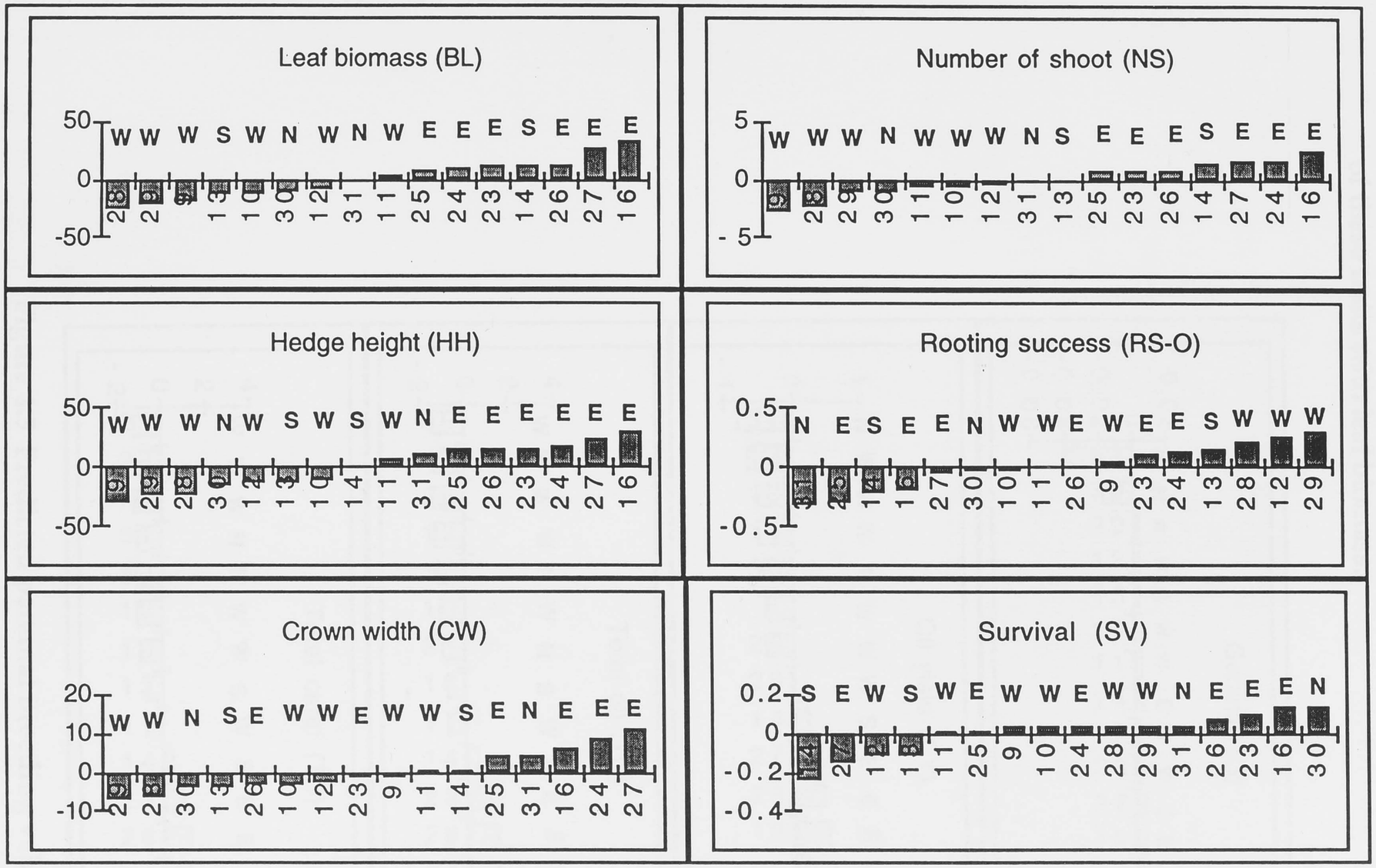

Figure 4.4 Predicted parental breeding values for growth traits

Where: number 9 - 31 represent families 1009, 1010, 1011,..... 1031.

W - Woondum; S - South Maroochy River; E - Eumundi; N - Noosa National Park.

Parents 1009, 1010, 1011, 1012, 1028, 1029, 1030 and 1031 had negative breeding values for most of the oil traits, with parents, 1009, 1012, 1028, 1029 the worst for total oil and total citral, and 1031 the worst for oil yield. The consistent pattern of parental performance for oil yield traits reflects in part the association of these traits with leaf biomass.

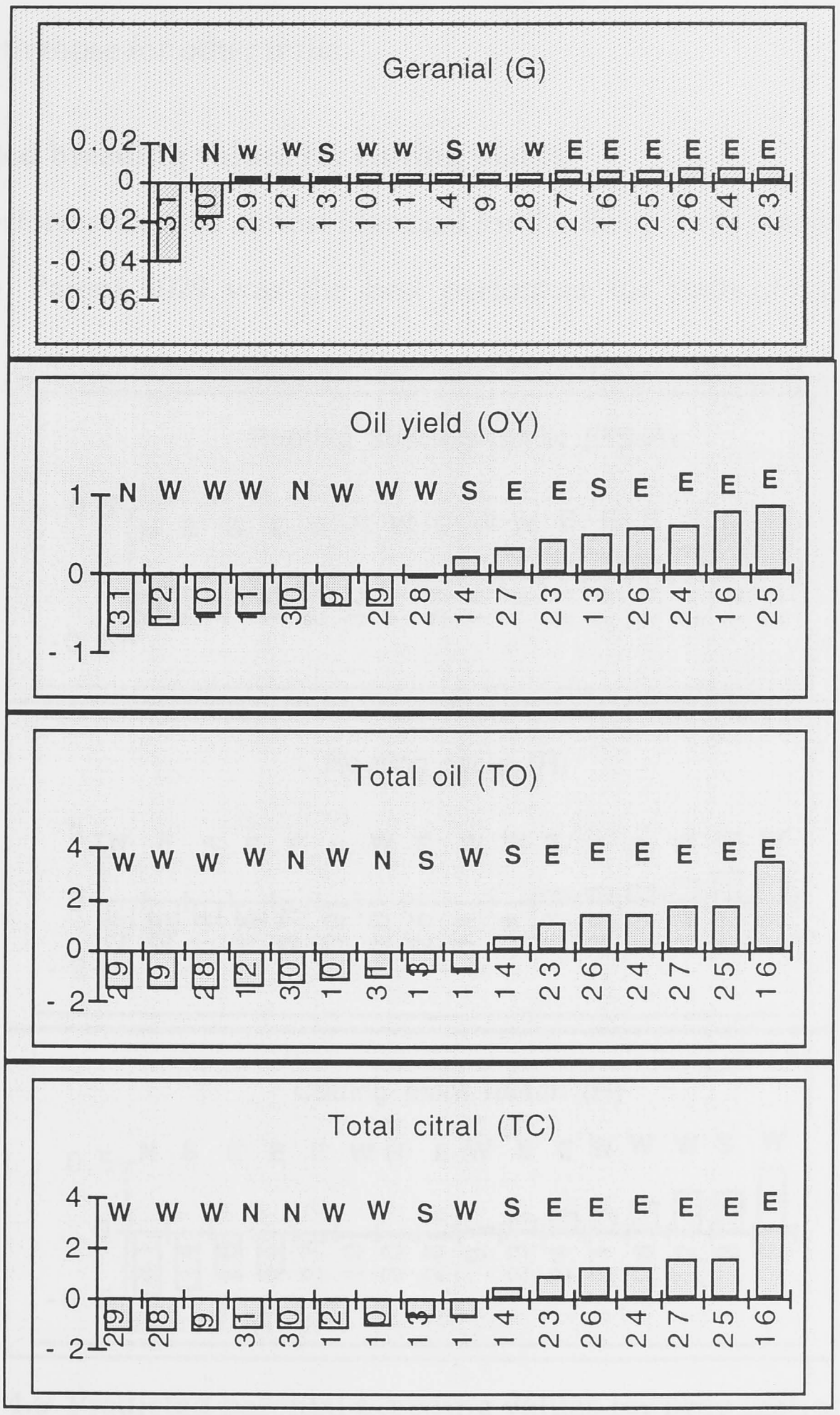

Figure 4.5 Predicted parental breeding values for oil traits

(3) Estimated breeding values for propagation traits

No general pattern was evident for the propagation traits of rooting success and survival. Specifically, parents 1029, 1028 and 1012 had the highest breeding values for rooting success (RS-O) and 1016 and 1030 were the best for survival (SV). Parents with poor breeding values for rooting success were 1031, 1025, 1014, 1016, 1027, 1030 and 1010, and those with negative breeding values for survival were 1014, 1027, 1012 and 1013 (Figure 4.4). These results contrast strongly with those for other traits.

(4) Estimated breeding values for cutting plants

Parental performance was almost identical for the three traits assessed in Trial 2 (Figure 4.6). Parent 1029 was the best performer for traits of rooting success

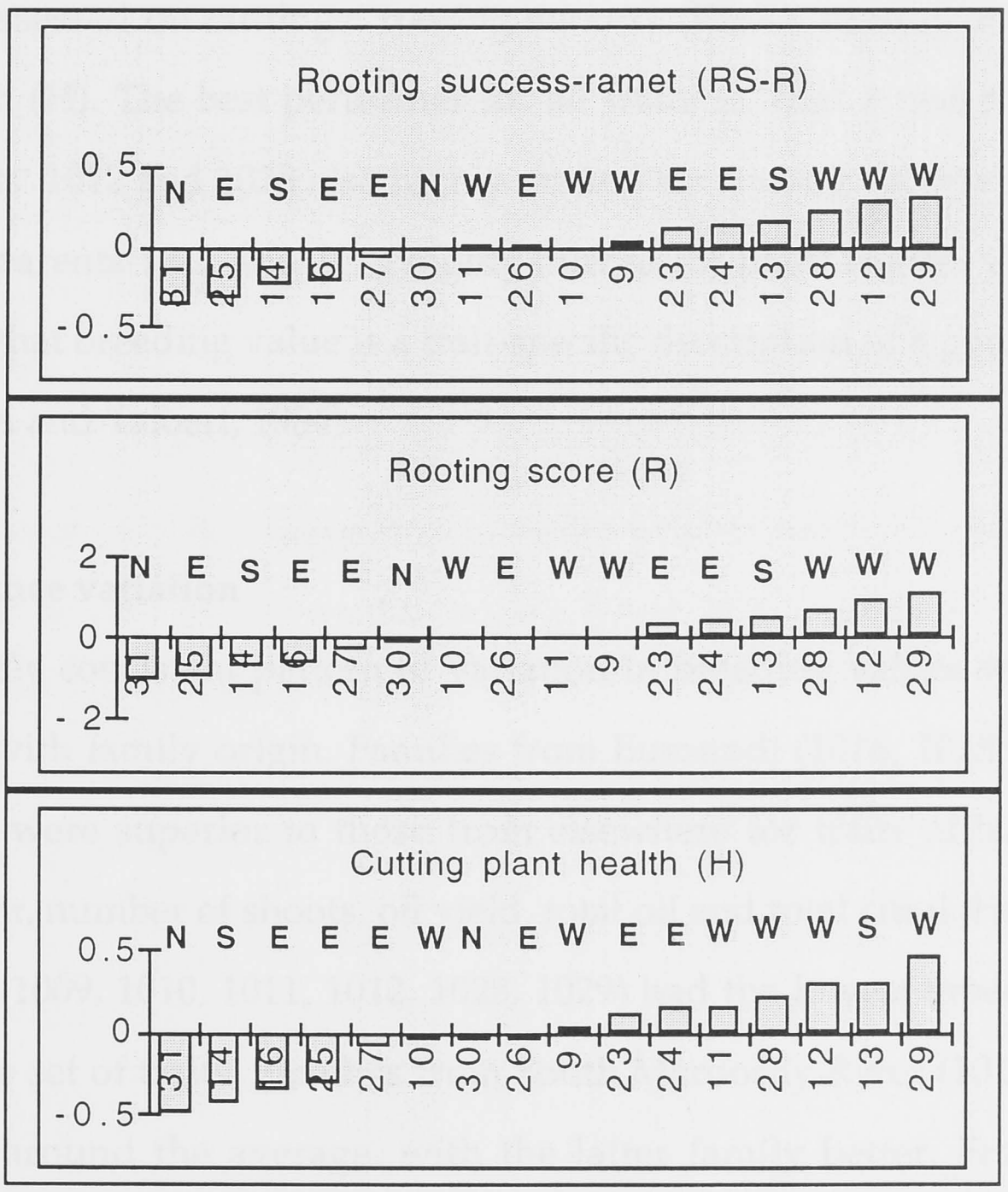

Figure 4.6 Predicted parental breeding values for propagation traits

(RS-R), rooting score (R) and cutting plant health (H). Parents 1012, 1013, 1028 and 1023, 1024 also performed well; parents 1025 and 1031 had the lowest

breeding values for RS-R and R, and 1031 and 1014 had the lowest breeding value for H (Figure 4.6 and Table 4.5).

In conclusion, parents 1014, 1016, 1023, 1024, 1025, 1026 and 1027 had consistently superior breeding values for growth traits; parents 1016 and 1027 were the best. The same group of parents showed positive breeding values for oil traits, although with slight variation in rankings. Parents 1025 and 1016 were best for citral yield and oil yield, and parent 1016 was superior for total citral and total oil. A different group of parents - 1012, 1013, 1028 and 1029 - were superior for RS-O, and parents 1030 and 1016 had highest breeding values for survival. Six parents - 1029, 1012, 1028, 1013, 1024 and 1023 - were superior for the traits assessed on cuttings: rooting success (RS-R), rooting score (R) and plant health (H). The best performer for all traits in Trial 2 was parent 1029 , with parents 1012 and 1028 also highly ranked for rooting success and rooting score, and parents 1012 and 1013 highly ranked for plant health. These results emphasize that breeding value is a trait-specific description of a parent's genetic value (Zobel and Talbert, 1984).

4.6 Provenance variation

The relatively consistent pattern of variation in breeding values was strongly associated with family origin. Families from Eumundi (1016, 1023, 1024, 1025, 1026, 1027) were superior to those from elsewhere for traits of leaf biomass, hedge height, number of shoots, oil yield, total oil and total citral. Families from Woondum (1009, 1010, 1011, 1012, 1028, 1029) had the lowest breeding values for the same set of traits. Families from South Maroochy River (1013 and 1014) performed around the average, with the latter family better. Families from Noosa National Park (1030, 1031) were below the average for most traits, with the latter family better.

In contrast, families with highest breeding values for rooting success were those from Woondum (1009, 1012, 1013, 1028 and 1029). Among the families from Eumundi, only 1023 and 1024 were above average for this trait. Of the South Maroochy River families, 1013 was above and 1014 below the average. One of the families from Noosa National Park (1031) had the lowest breeding value, and the other (1030) was slightly below average.

For survival, no pattern of provenance variation was evident. The best families (1030, 1016) came from Noosa National Park and Eumundi respectively, while the lowest family was 1014 from South Maroochy River.

4.7 Discussion

4.7.1 Comparison with previous reports

Differences in these results for oil trays with those reported for natural stands may reflect ontogenetic differences (*i.e.* these plants were young hedges) as well as the possible effects of environmental and analytical variation (*i.e.* stage of growth season, type of analysis and extraction, or storage conditions of samples). The photo - and thermal - instability of citral causes decomposition of the oil under certain conditions (Southwell, 1996), and may also be responsible for some of the differences observed.

The coefficients of variation show that large phenotypic variation was evident in total oil yield, total citral yield, leaf biomass, hedge height, number of shoots and crown width. The phenotypic variation in these traits may be partly due to the variation in individual plant quality at the time of establishing Trial 1. For example, the variation in seedling health and root system development when transplanted may consequently influence a seedling's ability to compete in the field, thus affecting leaf biomass yield and other growth traits, and then total oil and total citral yield.

Rooting success achieved in this project was relatively low compared with previous research results. There are several possible reasons for this. The first reason, and perhaps the most important one, is due to a difference in growing media which became evident only afterthe trial was established. In a preliminary study, a potting mix of 3:2 vermiculite - perlite was used, while the growing medium used in this project was a mix of 1:1 peat moss and washed river sand. There is much evidence to suggest that the development of containerized seedlings vary greatly with growing media. Phipps (1974) reported that peat and vermiculite in proper proportions yield the best result in seedling growth and root development. In Phipps' study on growing media, a mix of peat - sand (1:1) produced poor results in stem length, stem weight, diameter and root weight, and ranked as the seventh of the nine media under test. The superiority of peat - vermiculite may be due to a high cation exchange and water holding capacity, their relative freedom from insects and diseases, and the better conditions of aeration and drainage for plant growth (Tinus and McDonald, 1979).

The number of nodes on cuttings and node location may be another important contributing factor. Cuttings with two to four nodes are the general practice for *Eucalypt* species. In a study of *Eucalyptus camaldulensis*, cuttings with four nodes were significantly higher in rooting success than those with two nodes (Geary and Harding, 1984). Regarding node location, a node within two cm of the cut base resulted in best rooting for sycamore (Land *et al.*, 1991). However, most of cuttings used in this project had two nodes, and some were prepared with one node only, as a uniform cutting length (8 cm) was used and large plants had a longer internode than 8 cm. The better response of rooting to the number of nodes may be attributed to higher concentrations of root promoting chemicals and carbohydrates in nodes than in the stems (Smith and Wareing, 1972; Hartmann *et al.*, 1990).

Another possible cause of poor rooting may be due to the effect of the onset of dormancy. For softwood cuttings, rooting success is best when cuttings are collected early in the growing season (Dirr and Heuser, 1987; Hartmann *et al.*, 1990). Unfortunately, the cuttings used for this project could not be collected earlier than May, which is late autumn in Queensland. Increasing lignification and concentrations of root-inhibiting growth regulators in later collections may be an important contributor to this relatively low striking rate (McComb and Wroth, 1986; Paton *et al.*, 1981).

Other technical factors involved in the setting and tending processes may also be important sources of variation in rooting success. In general, it is not possible to draw precise conclusions from a single-trial data, and great potential for improvement can be expected through further research and improved techniques.

4.7.2 Genetic variation

Heritability estimates showed that most of traits were under a very strong genetic control. However, neral, geranial and citral displayed no additive genetic variation. It seems that these traits may be influenced by genes with major effects. Although heritabilities for oil yield, total oil, total citral and rooting success were obviously overestimated, they indicated that gains can be achieved through selection at an individual tree level. Predicted breeding values for all traits, other than those for neral, geranial and citral, reflected an obvious difference between families, suggesting a great potential for improvement through family selection.

4.7.3 Correlations between traits

The high correlation between leaf biomass and oil yield traits suggests indirect selection on biomass will be effective in increasing oil production. To determine oil yield per plant or per unit area, leaf biomass must be measured or estimated.

This is a difficult and time-consuming task. The highly positive correlation between leaf biomass and hedge height and number of shoots suggested potential for use of hedge height and number of shoots in calibrations for leaf biomass.

The moderate negative correlation between rooting success and growth traits and oil traits is an important concern. It is apparent that rooting success had a negative correlations (-0.56) of similar magnitude with leaf biomass, hedge height and crown width, and these relationships are considered more important than those with oil traits as total oil yield and total citral yield depend on biomass production. This trend could possibly be explained by the following reasons:

- The first may be due to the effects of ontogenetic age of the meristem (Preece *et al.*, 1991) and physiological ageing (Hare and Land, 1982). As larger plants tend to be higher and have wider crown and longer shoots, cuttings from the biggest hedges may have been more distant from the roots, and so perhaps more mature than those collected from smaller plants (Dr. M. J. Dieters, pers. comm.).

- Another possible cause may be, as mentioned earlier in **4.6.1**, due to the location and number of nodes. As larger plants tend to produce shoots with longer internodes, greater than 8 cm, more cuttings from the bigger plants were prepared with one node about 6 cm away from the cutting base. The difference of the concentrations of carbohydrates and root promoting chemicals in the nodes and in the stems may cause this variability in rooting ability.

- Technical factors involved in the cutting collection, setting and tending processes may be one of the most important aspects which need more investigation and research.

In conclusion, there is significant variation in most traits at individual plant and family levels. The variation seems also to be strongly associated with family origin, and families of Eumundi origin deserve priority in forming breeding populations. In addition, improving rooting success may become a major concern for deployment, and the Woondum provenance appears superior in this trait. However, great potential for improving rooting success can also be expected through improved cultivation techniques.

CHAPTER 5. IMPLICATIONS FOR BREEDING STRATEGY

5.1 Introduction

The tree improver's principal role is to recognize genetic variation, isolate it, package it into desired phenotypes and multiply them (Zobel and Talbert, 1984), so as to maximize genetic gains in production populations. Although the emphasis in breeding objectives for *B. citriodora* may vary with breeding programs, maximum production of the commercially valuable component of the oil, citral, is the objective here, and likely to be relevant to any project breeding *B. citriodora* for oil production.

The development of an appropriate breeding strategy, incorporating sound selection and mating decisions, and an efficiently structured and conducted breeding program, are necessary if gains in the breeding objective are to be maximized. This Chapter discusses selection decisions and proposes a breeding strategy aimed at improving citral production, using information for *B. citriodora* presented in Chapter 4.

5.2 Basic concepts of tree breeding strategy

As with any species, successful breeding of *B. citriodora* depends on a sound breeding strategy. The processes of selection, testing, mating, multiplication and production are incorporated into a breeding strategy which typically consists of three major populations - the base, breeding and multiplication populations (White, 1987; Zobel and Talbert, 1984). The base population provides individuals which are tested and selected for the breeding population; the breeding population repackages these genetic resources to produce superior genotypes and generate further variation through mating; the multiplication population serves as a distributor for the deployment of superior stock. This breeding cycle is illustrated in Figure 5.1.

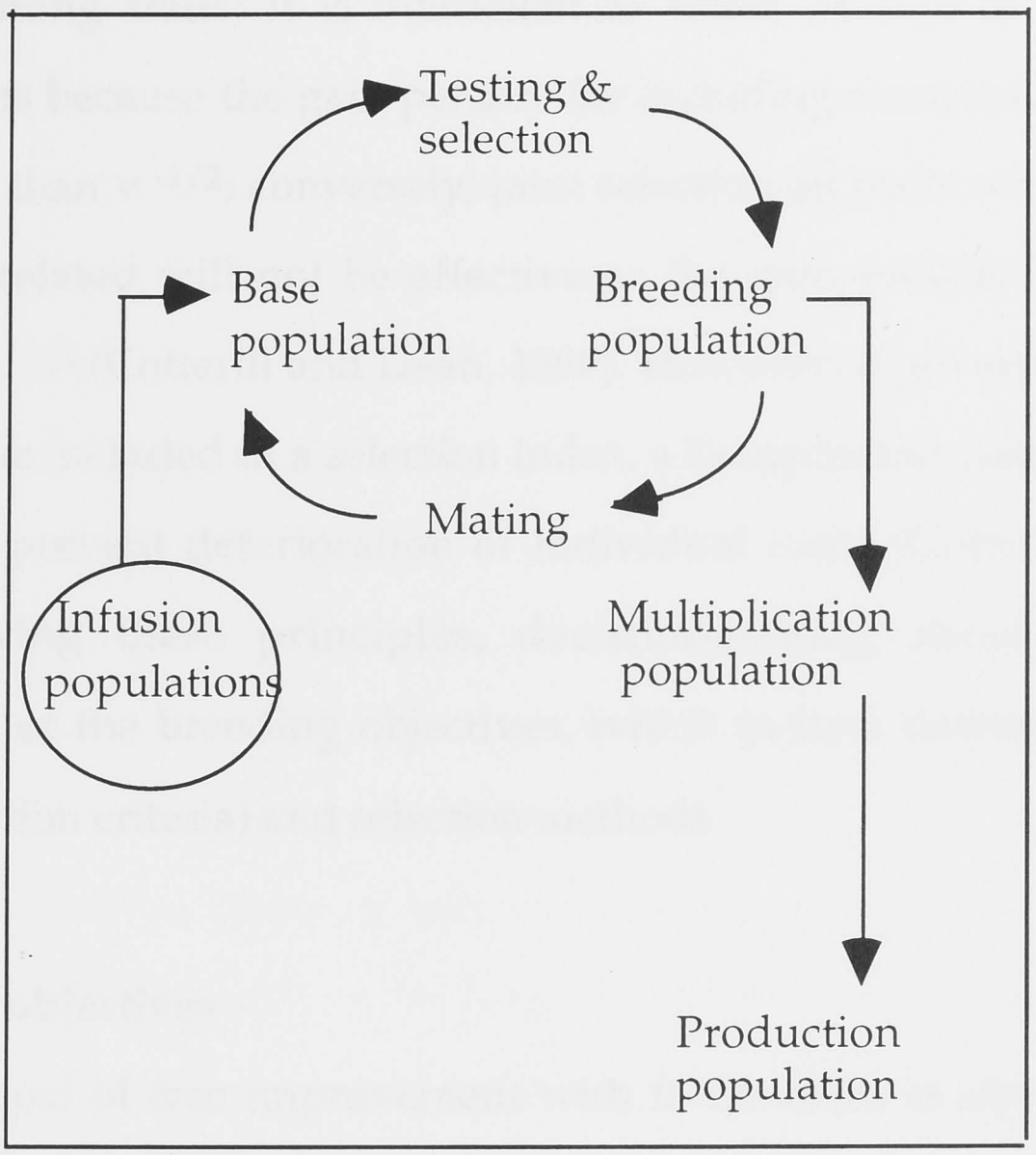

Figure 5.1 The tree breeding cycle

(Kanowski and Savill, 1991, adapted from White, 1987)

5.3 Selection

5.3.1 Principles of genetic selection

To make sound selection decisions, several basic principles of genetic selection should be kept in mind. First, selection is based upon the principle that the average genetic value of selected individuals should be higher than the population mean (Zobel and Talbert, 1984). This is achieved by accurate identification of the relative genetic worth in the characters of interest. Secondly, selection should be focused on those traits of high economic importance. The number of traits under selection should be reduced to the practical minimum because the genetic gains in individual traits will almost certainly diminish as the number of traits under selection is increased; under multi-trait selection, the gain expected in any one trait is only $v^{-1/2}$, where v is the number of traits under selection, of that from selection on a single character alone (Cotterill and Dean, 1990). Finally, attention should be given to the

correlation among traits; it is inefficient to select on two or more highly correlated traits because the gain penalty for including more traits in selection would be less than $v^{-1/2}$; conversely, joint selection on traits which are highly adversely correlated will not be effective as the gain penalty would be far greater than $v^{-1/2}$ (Cotterill and Dean, 1990). However, if adversely correlated traits have to be included in a selection index, a Kempthorne restriction should be applied to prevent deterioration of individual traits (Cotterill and Dean, 1990). Following these principles, decision-making should start with determination of the breeding objectives, which in turn determine traits for selection (selection criteria) and selection methods.

5.3.2 Breeding objectives

The ultimate goal of tree improvement with *B. citriodora* is assumed to be to increase the profitability of growing this species for citral production. Achieving this breeding objective will require that genotypes with high citral yield be propagated successfully, either by seedlings or cuttings, and managed to maximize citral production. The relative costs and gains from use of cuttings will determine whether they are the preferred means of deployment.

5.3.3 Selection criteria

The high variation in oil and growth traits, both between and within families, the high heritability estimates for these traits, and the strong positive correlations among some of them provide good prospects for genetic improvement. However, the breeding program needs to balance both immediate and long term gains. Immediate gains from deployment are dependent on propagation success by either cuttings or seedlings; longer term gains depend on a good strategy to generate, extend and use variation.

Amongst the oil traits assessed here (Table 3.2), total citral is the obvious choice as a selection criterion. It is a direct measure of the product of interest,

integrating citral content, oil yield and leaf biomass (Equation 3.5). Given the primacy of leaf biomass in determining citral production, and its incorporation in the calculation of total citral, it is not necessary to use leaf biomass as a selection criterion. Selection for total citral will increase biomass production through correlated response. Additionally, it may be possible to improve leaf biomass by silvicultural practice.

Coppicing ability (measured by the number of shoots, NS) is also an important selection criterion. Plantations of *B. citriodora* for oil production are likely to be managed as coppice, exploiting the natural ability of the stump and root system to produce successive crops of leaf after harvesting. Coppice vigour will therefore have a major impact on the long-term productivity of plantations, and thus coppicing ability must be an important criterion for selection.

The propagation traits, survival rate of seedlings and rooting success of cuttings, are critically important for the successful development of plantations of *B. citriodora*. Survival rate had very weak correlations with oil traits, indicating that selection for increasing survival rate is unlikely to affect oil yield on an individual plant basis. However, higher survival implies higher oil production per unit area. The moderately negative correlation between rooting success (RS-O) and total citral shows that it is difficult to improve both traits simultaneously using recurrent selection (Eldridge *et al.*, 1994). Whilst the high heritability of RS-O suggested that good gains can be made by selection, breeding programs will need to balance gains in this trait with those in citral yield, as propagation success is fundamental for successful deployment of cuttings of superior genotypes. Additionally, evidence from a preliminary study on rooting success of *B. citriodora* suggests that rooting success could be greatly improved by cultivation techniques (S. Walker, pers. comm.).

The results reported in Chapter 4 also demonstrate that provenance differences should be taken into account in making selection decisions. In general, families from Eumundi perform better than those from other provenances in terms of selection criteria other than rooting success. Conversely, families from Woondum have high rooting success and families from Noosa National Park have high survival. Thus, selection to improve oil production in *B. citriodora* could be focused on good performers from Eumundi for improving total citral, and on good families from Woondum for improving rooting success. Improvement in both total citral and rooting success might be achieved by crossing parents from the two provenances, although this has yet to be verified. Families from Noosa could be used similarly to enhance survival if necessary.

In summary, total citral, coppicing ability, survival rate and rooting success are appropriate selection criteria for the improvement of oil production in *B. citriodora*, and gains should be maximized by focusing selection effort on these traits.

5.3.4 Selection methods

A suitable combination of selection method and mating design is the foundation of a breeding program (Cotterill, 1984), and maximizing genetic gains from advanced breeding programs is largely a matter of efficient selection (Cotterill and Dean, 1990). There are a number of selection methods which can be applied in various situations; the most common are tandem selection, independent culling, and the use of either base, primary, Elston or Smith-Hazel indices.

The Smith-Hazel index is preferred because it takes account of the economic importance of traits, their heritability and correlations among them, uses information from relatives, allows the use of restrictions to prevent deterioration in adversely correlated traits, and incorporates juvenile-mature

correlations in the definition of genetic worth so as to maximize the efficiency of selection (Cotterill and Dean, 1990). However, it requires reliable estimates of genetic parameters. If the available estimates of heritability and genetic correlations are unreliable, the predicted gains will also be unreliable, and hence the selection efficiency will be reduced to a level approaching that of the base index (Cotterill and Dean, 1990). Further, if information concerning the relative economic weight of traits is not available, incorrect decisions could be made. Given the unreliability of parameter estimates here, the lack of economic information, and the early stage of breeding *B. citriodora*, the use of index selection seems inappropriate here. Independent culling was considered to be a more appropriate alternative in this case. Although independent culling cannot easily accommodate multi-trait information, it has the advantages of flexibility and simplicity (Cotterill and Dean, 1990). It also requires less genetic information, appropriate for the quality of estimates and stage of breeding here.

Under independent culling, parental breeding values, which are more reliably estimated than heritabilities and genetic correlations, are the basis of selection decisions. At this stage of breeding *B. citriodora*, selection at the family level is more appropriate than selection on an individual tree basis, although outstanding individuals could be selected for immediate deployment. Thus, selection decisions here were based on family performance in the traits identified above as being most important. Family performance for various pairs of these traits is graphed in Figures 5.2 - 5.4.

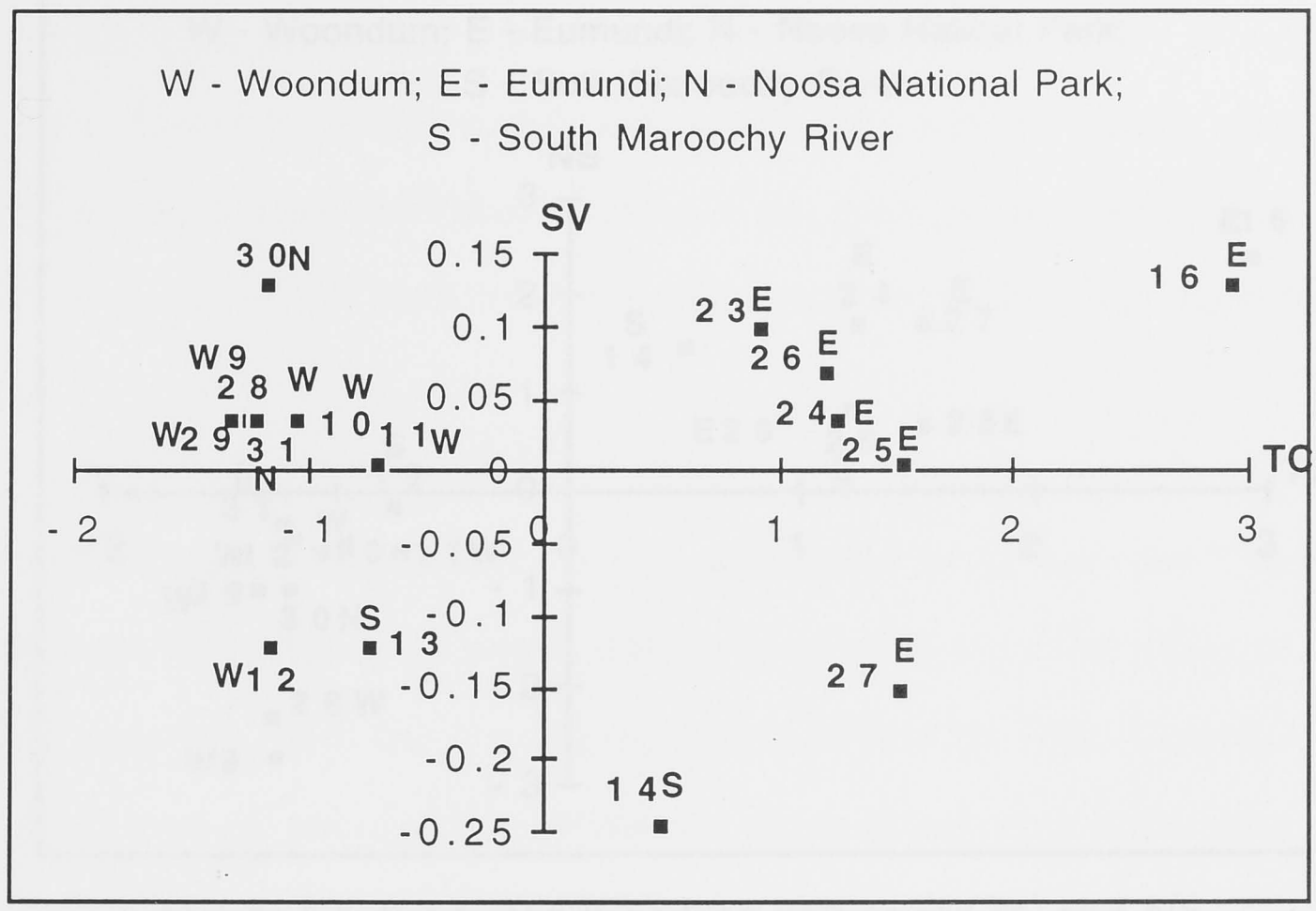

Figure 5.2 0 Predicted breeding values for TC and SV

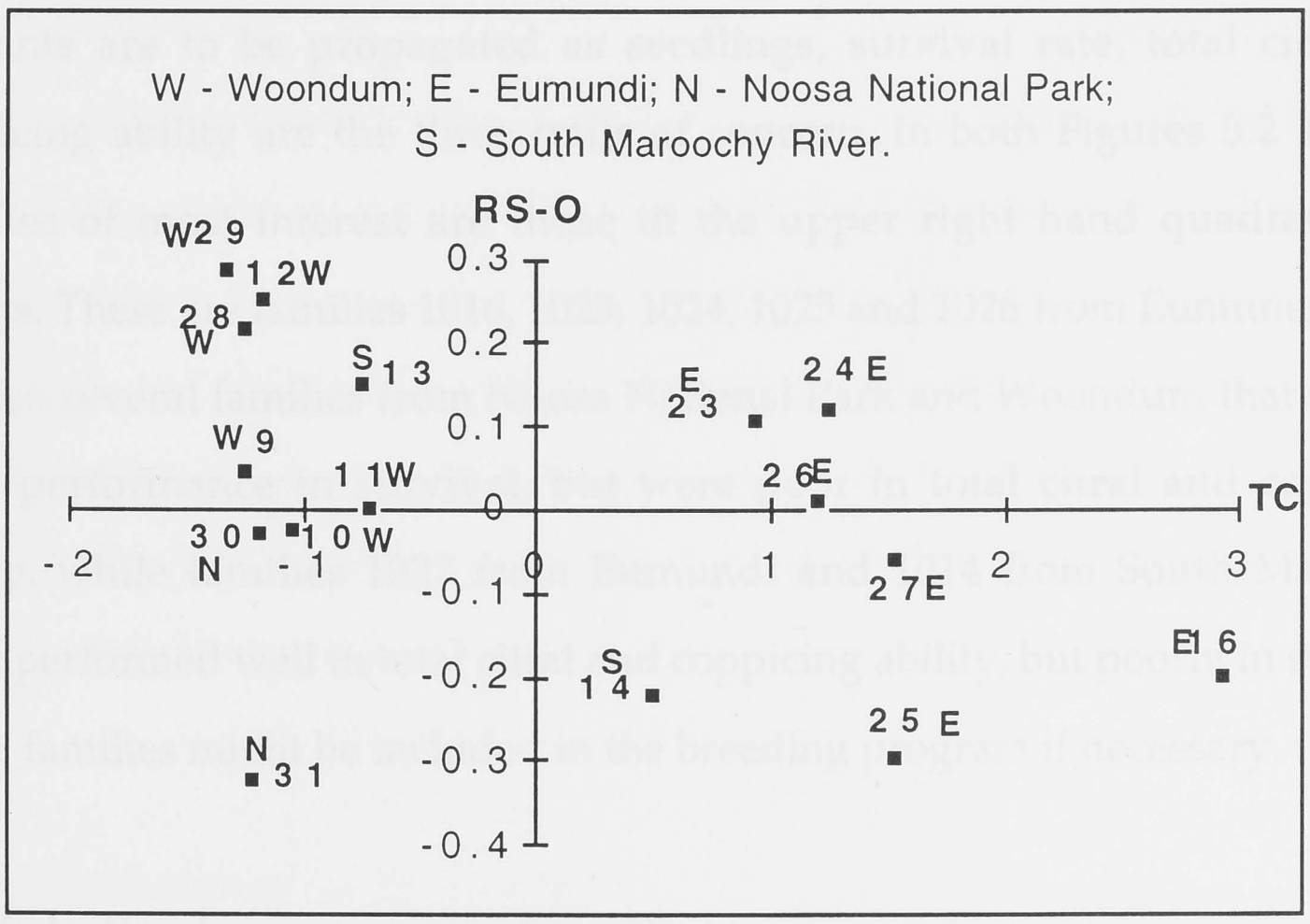

Figure 5.3 Predicted breeding values for TC and RS-O

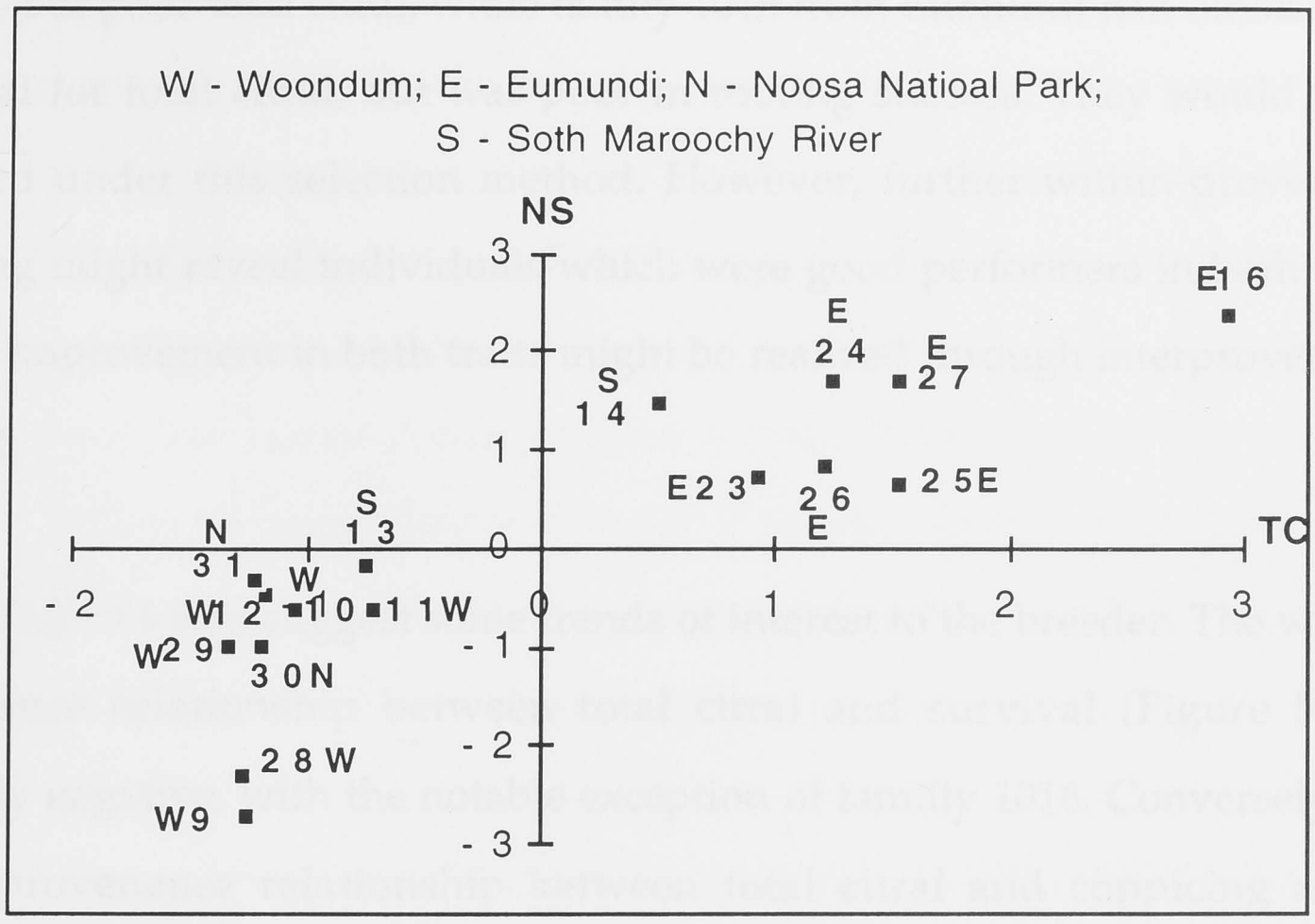

Figure 5.4 Predicted breeding values for TC and NS

5.3.5 Selection for propagation by seedlings

If plants are to be propagated as seedlings, survival rate, total citral and coppicing ability are the three traits of concern. In both Figures 5.2 and 5.4, families of most interest are those in the upper right hand quadrat of the graphs. These are families 1016, 1023, 1024, 1025 and 1026 from Eumundi. There are also several families from Noosa National Park and Woondum that showed good performance in survival, but were poor in total citral and coppicing ability, while families 1027 from Eumundi and 1014 from South Maroochy River performed well in total citral and coppicing ability, but poorly in survival. These families might be included in the breeding program if necessary.

5.3.6 Selection for propagation by cuttings

If vegetative propagation is to be employed as the deployment strategy, the appropriate combination of traits for selection would be rooting success of cuttings, total citral and coppicing ability. Families which meet these selection criteria (Figures 5.3 and 5.4) are 1023, 1024 and 1025 from Eumundi. Three families (1029, 1012 and 1028) from Woondum showed excellent rooting

success, but poor total citral, while family 1016 from Eumundi had outstanding potential for total citral, but was poor in rooting success. They would be all excluded under this selection method. However, further within-provenance sampling might reveal individuals which were good performers in both traits, or joint improvement in both traits might be realized through interprovenance crossing.

Figures 5.2 - 5.4 also suggest some trends of interest to the breeder. The within-provenance relationship between total citral and survival (Figure 5.2) is generally negative, with the notable exception of familiy 1016. Conversely, the within-provenance relationship between total citral and coppicing ability (Figure 5.4) is generally positive. The variation in total citral associated with any given level of the rooting success of cuttings (Figure 5.3) suggests that it is possible to improve the former with relatively little effect on the latter.

To summarize, immediate deployment should be focused on families from Eumundi, especially on those (1023, 1024 and 1026) that perform well in all the traits of interest. These families should also form part of the main breeding population. In addition, a specific breeding program may be needed to incorporate families such as 1016, the best performer for total citral and coppice, and 1029, superior in rooting success, in a population of superior genotypes which advance the two traits together.

5.4 Breeding strategy for *B. citriodora*

5.4.1 Constraints to breeding

Besides reliable genetic information, a number of factors will influence the choice of the most efficient and cost-effective breeding strategy. The major factors here are the availability of genetic resources, information on reproductive biology, and cultivation technology.

(1) Availability of genetic resources

All tree improvement programs must have mating and seed production at some stage of their development if continued gains are to be achieved (Zobel and Talbert, 1984). Although natural populations of *B. citriodora* are limited and may to some extent impose constraints on the scale of breeding, the availability of genetic resources is not considered as a major constraint to the breeding strategy proposed here. However, lack of reliable genetic information and uncertainty about utility of the existing genetic resources will be one of the obstacles to the success of a breeding program.

Although useful information about provenance and family variation in traits of interest is now available as a result of the work described here, genetic parameter estimates remain too poor, and population size too small, to be good bases for subsequent breeding. Therefore, the breeding strategy must include further testing, of a broader range of provenances and larger number of families, across the range of sites typical of those on which *B. citriodora* plantations are likely to be grown. This implies more intense sampling from throughout the range of the species. The rare Chemotype "2" is obviously of interest to growers and breeders. However, further investigation of the properties of the individuals identified in this work will be necessary before the best strategy for their use can be decided.

(2) Lack of information on reproductive biology

Knowledge of the reproductive mechanisms, potential for sexual and asexual reproduction, extent of variation and the heritability of these characteristics are essential to developing a breeding strategy (Zobel and Talbert, 1984). This information is required to determine how to collect and store seed and pollen, whether seedling or vegetative propagation should be used, whether control or open pollination should be practised, and the appropriate coefficient of relationship for estimation of genetic parameters. Information on these

biological aspects of *B. citriodora* is still incomplete and will affect the options available for developing a suitable strategy.

(3) Cultivation technologies for this species

Cultivation techniques are another important aspect of successful tree breeding. Such simple things as how to improve seed germination, how to grow seedlings in the nursery, and how to outplant the trees and care for the plantations must be determined for a breeding program to succeed in improving plantation production (Zobel and Talbert, 1984). Unfortunately, only very limited information on these aspects is so far available for *B. citriodora*. However, there is adequate information for commercial success, and to identify priorities - *eg* propagation techniques - for further research.

5.4.2 Breeding and deployment strategies for improving oil and propagation traits

The breeding strategy proposed here was based on the genetic information available from one progeny test and one clonal trial of 16 families representing four of the 12 provenances. The strategy comprises three parallel and interdependent strands of activity:

1. breeding of a main population;
2. deployment of superior genotypes as soon as they become available;
3. interprovenance hybridisation to improve both total citral and rooting success.

The strategy is represented in Figure 5.5 and detailed below.

5.4.2.1 Breeding strategy for long-term improvement

(1) Establishment of base population

The principal genetic resources available for the *B. citriodora* improvement program are natural populations of 12 identified provenances described in **2.1.2**. There is as yet no detailed knowledge of the occurrence of individuals in these disjunct populations. Only limited genetic information is available from

the 16 families of four of the 12 provenances tested in this project and the size of the progeny test population is too small to form a reasonable genetic base for a breeding program. Theoretically, the base population should be large enough to include adequate genetic diversity, to meet selection requirements (Zobel and Talbert, 1984), and to allow reliable estimates of genetic parameters (Cotterill and Dean, 1990). Consequently, base populations often contain hundreds to many thousands of genotypes (Zobel and Talbert 1984). Unfortunately, the disjunct distribution of small populations and the difficulties of collecting seeds of *B. citriodora* in the wild impose some constraints on meeting these goals.

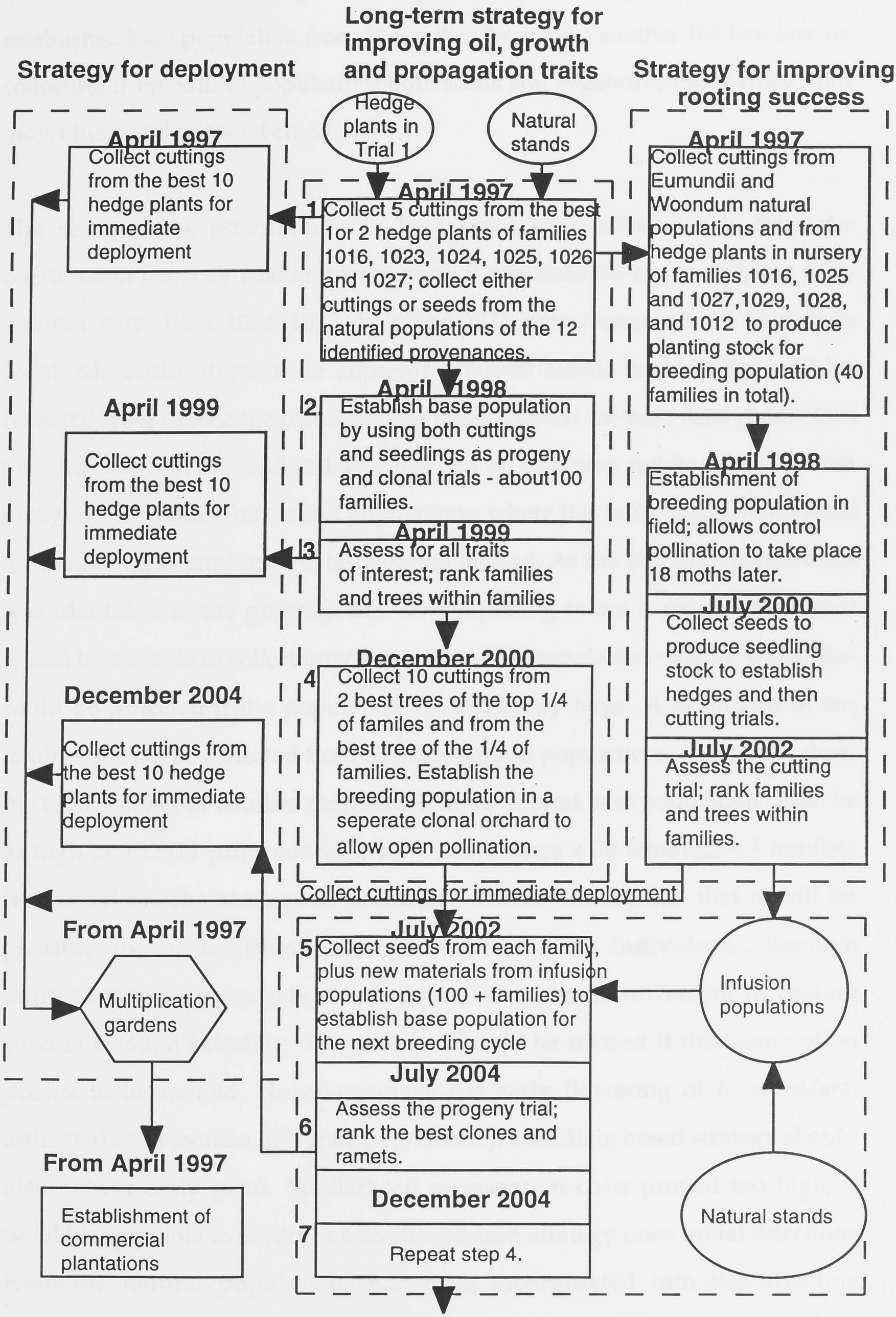

Figure 5.5 Proposed breeding strategy for *B. citriodora*

It is considered feasible (J. Doran, pers comm.) to expand the currently-established base population from 16 families by at least another 100 families, by collecting from natural populations both seeds and vegetative propagules from individuals without seed crops.

The second base population can be formed from selections in both the Beerburrum progeny trial and from natural populations. In the progeny trial, families 1016, 1023, 1024, 1025, 1026 and 1027 from Eumundi and 1014 from South Maroochy River were superior for total citral. Ten cuttings will be collected from two hedge plants of each family. Given the poor seed production of *B. citriodora* in the wild (**2.1.4**), collection of seed may not be possible from many individuals in the natural populations; where it is not, vegetative material for the setting of cuttings will be collected instead. As the Eumundi provenance was identified in the progeny trial as comprising many superior families, it would be sensible to collect intensively from this population - up to 30 families could be collected if the population is sufficiently large. A minimum of ten families should be collected from all other natural populations, if possible; thus, the total number of families represented in the second base population could be as high as 150 (11 provenances x 10, 1 provenance x 30 families, + 7 families from Trial 1). The strategy is predicated on the assumption that it will be possible to set sufficient cuttings from selected individuals, through improvement in propagation techniques (**4.1**) and improvement of rooting success through breeding. The strategy should be revised if this assumption proves to be invalid. However, given the early flowering of *B. citriodora*, estimated at 18 months (J. Doran, pers comm.), a seedling based strategy should also deliver early gains. Similarly, if propagation costs proved too high, it would be possible to revert to a seedling-based strategy once initial selections from the natural populations had been incorporated into the breeding population. The correlation between seedling and cutting performance, and the

feasibility of control pollination, will also require verification under this strategy.

Two separate but adjacent or interplanted trials, one of seedlings and the other of cuttings, would be established as a result of these collections. The trials would be replicated across sites if possible and as necessary to sample the range of production sites. A minimum of either 30 healthy seedlings or cuttings will be randomly chosen from each of the families. Although a randomized complete block design was used in Trial 1, a balanced incomplete block design (Williams and Matheson 1994) would be preferable in these tests. The stock plants will be cut back to 30 cm eight months after planting. Survival rate will be assessed in conjunction with the hedging operation. Cuttings collected from the hedging operation will be set in the glasshouse. Eight weeks later, cutting stock will be assessed for rooting success. The overall assessment of the progeny test for oil, coppice and growth traits will be conducted one year after planting the experiment.

(2) Establishment of breeding population

After assessment, cuttings will be taken from selections in the progeny test and established on a separate site, to allow the trial to continue to be used for cuttings production. Parameter estimates should be good enough to allow the use of combined index selection. Given the early stage of the breeding program, culling within families can be heavy, but culling of families should be relatively light. In practice, 10 cuttings from each of the best 2 trees of the top 1/4 of families, from the best tree of the second best 1/4 of families, and from outstanding individuals in other families, will be collected and cultivated in the nursery for one year. These cutting plants will be then transplanted to a separate clonal orchard to allow open pollination eighteen months after establishment. The orchard will be designed to maximizing outcrossing. The population size will be thus kept at around half the original number of families.

(3) Establishment of multiplication population

Heavy culling intensity will be applied to the breeding population, based on the assessment of the second progeny test in July 2005. Cuttings will be collected from the best 10 individuals within the breeding population to establish the multiplication population, by either seed or cuttings production, depending on the relative costs. Ten individuals should be adequate if deployment is by cuttings; around 30 would be necessary if seedlings are to be produced for deployment.

(4) Progeny test and selection for the next breeding population

Seedling production for the next progeny trial will start with seeds collected from the previous breeding population in July 2002, and the progeny trial will be established by using one-year-old seedlings in July 2003. Seedlings will be hedged to 30 cm eight months after planting for assessment of coppicing ability and rooting success of cuttings. The progeny test will be assessed in July 2004. The activities described in **5.4.2.1 (2)**, plus new materials from infusion populations, will be used to establish the base population for the next breeding cycle.

5.4.2.2 Immediate deployment to meet the demand of growers

Currently, there are number of local growers awaiting the availability of high-quality planting stock. In order to meet their demand and promote the development of the industry, a quick and practical approach is the direct deployment of the best individuals identified at any stage of the breeding cycle. The strategy for deployment is a vegetative propagation approach with rooted cuttings. Propagules will be collected from the best 10 individuals of families with high citral production and good rooting success and cultivated in the nursery for one year. Fully-developed cutting plants will then be transplanted in a multiplication garden and hedged to 30 cm eight months after planting to

promote coppice production. Successive hedging operations will be conducted at four-month intervals to ensure the production of planting stock throughout the year.

The first deployment will start in April 1997, in conjunction with the long-term strategy for improving oil, growth and propagation traits. Cuttings will be collected from the best 10 hedge plants identified in the first progeny test. The second deployment will begin in April 1999 when the second progeny test is assessed. The third deployment will start in July 2002 when selections are available from the interprovenance hybrid population, and the next will be carried out when the third progeny test is evaluated in December 2004. The deployment cycle will continue subsequently in the same manner.

5.4.2.3 Strategy for improving rooting success

As rooting success is negatively correlated with other selection traits, the inclusion of rooting success as a selection criterion will diminish the rate of improvement in other traits. Rooting success is critical to the success of a cutting-based breeding strategy and commercial plantations. One approach to this problem is to establish a separate breeding line for improving this trait, in parallel with the main breeding program outlined above.

The results of the first progeny test identified the Eumundi provenance as best for total citral, and the Woondum provenance as best for rooting success. The strategy proposed here seeks to produce hybrids which incorporate these two characters.

The first step is to establish a breeding population based on the genetic information obtained from the first progeny test. Cuttings collection will be focused on the best trees in families 1016, 1023, 1024, 1025, 1026 and 1027 of Eumundi origin, the best families for total citral, and on the best trees in

families 1029, 1012 and 1028 of Woondum origin, the superior families for rooting success. In addition, individuals of family 1013, with high rooting success, and family 1014, with high total citral, from South Maroochy River may be included. In order to have a reasonable genetic base, additional cuttings will collected from individuals in natural populations of these provenances. The breeding population size will be targeted to have 40 families in total, with around 20 families from each of the Eumundi and Woondum provenances.

The breeding population will be established in the field by using one-year-old cutting plants in April 1998. An appropriate mating design - either a full or partial factorial, or double pair mating, depending on resources available and required - will be employed when plants start flowering eighteen months after planting. Seed collection for the establishment of hedge plants can be expected in July 2000. The cuttings trial for the assessment of rooting success will be established from hedges of one-year-old seedlings. These seedlings will be cut back to 30 cm eight months after planting in the field, and cuttings will then be collected from these hedge plants in January 2002 and set in a glasshouse. Rooting success will be assessed in conjunction with the assessment of the ortets for total citral production in July 2002. The superior genotypes identified in this cutting test will be incorporated into the main breeding line for the next breeding cycle by collecting seeds for inclusion in the base population, and incorporated into the deployment program by collecting cuttings for multiplication.

This strategy combines the deployment, long-term improvement and joint trait improvement together in a single breeding program. In this strategy, the base and breeding populations serve sequentially as bases for progeny testing, selection and deployment. The strategy requires a high level of skill and efficiency in establishment and assessment of tests, and in data analysis, as cuttings and seedlings will be used together throughout the breeding cycle.

However, the strategy is a practical means to address the both short and long-term needs of this new industry.

5.5 Conclusions and recommendations

B. citriodora is a most promising tree species of tropical rainforests for citral production. The wide uses of its end product - citral - in aromatherapy, perfumery, food and drinking flavouring and medicines indicate a great potential in world markets; for example, the world demand for *B. citriodora* spice is predicted to be 3000 tonnes per annum by the year 2010. Fresh leaf of *B. citriodora* currently trades at $12/kg, and oil at $600/l; the price of oil is expected to stabilize at around $ 100/l (J. Doran, pers. comm.). In addition, the reproductive and ornamental features of this species offer potential for urban and agro-forestry. Growers may expect a broad range of attractive returns from the cultivation of *B. citriodora*.

The goal of the study reported here was to gather genetic information for the establishment of populations to meet the demand of commercial plantations for superior planting stock. Specific aspects of the study included the estimation of genetic parameters in oil, growth and propagation traits, and consideration of the implications of these genetic parameters for breeding and propagation strategies. The major results of the project are summarized below.

5.5.1 Ethanol extraction method

Although the ethanol extraction method has been applied to oil analysis of many tree species, its accuracy may vary with oils under test, and calibration against the standard method - steam distillation - is therefore necessary when it is applied to a new species. Results from comparison of the two methods and the time regime for extraction in this project showed no significant differences in citral concentration and oil yield between ethanol extraction and steam distillation, and between two-week and four-week extraction periods. These

results suggested that the ethanol extraction method could be a reliable means of estimating citral concentration and yield for *B. citriodora*. However, further testing and development of this methodology is recommended, as the calibration made in this study was based on a very limited samples.

5.5.2 Genetic variation in oil, growth and propagation traits

The experimental population yielded, on average, leaf-oil in sufficient quantity (2.6% of fresh leaf; 4.5% of dry leaf) and suitable quality (85% citral) for use as commercial sources of *B. citriodora* oil. Variance analysis showed that there were significant difference between and within families in most traits of interest, e.g. total citral, leaf biomass, coppicing ability, survival and rooting success. From analysis of breeding values, family 1016 ranked as the best performer for total citral, leaf biomass, and coppicing ability; family 1029 was identified as the best for rooting success; and family 1030 as best for survival. The results also reflected the variation between provenances. The Eumundi population was superior for all traits other than rooting success, the Woondum population was superior for rooting success, and the Noosa National Park population was best for survival. Clearly, the variation evident between and within families as well as amongst provenances allows significant improvement in citral production by selection and breeding.

5.5.3 Genetic parameters

Narrow-sense heritabilities for oil traits, with the exception of those for concentrations of neral, geranial and citral, were high (>1) and probably overestimated, due to the relatively small data set and the arbitrary use of 1/2.5 as the coefficient of relationship. Heritability estimates for growth and propagation traits were moderate to high. Broad-sense heritabilities for propagation traits in Trial 2 were moderate. Although these estimates are not precise, they suggest that most of traits concerned were under strong genetic control.

The genetic correlations between most oil and growth traits were large and positive, indicating good potential to focus selection efforts on one or two key traits without adverse effects on other traits of interest. However, survival had very weak correlations with all traits and can be considered as an independent character, while the negative correlations associated with rooting success (RS-O) imposes a constraint on the development of breeding strategies for *B. citriodora*.

5.5.4 Some recommendations

Despite this and other preliminary work (House *et al.*, 1996), the genetic study of *B. citriodora* is still in its infancy. The information realized from the current research project is still not sufficient to establish a sound breeding program, as the population size of sixteen families is too small to produce reliable estimates of genetic parameters and to establish an adequate base population. Therefore, the following parallel studies are recommended to support the implementation of the proposed breeding program.

- Organize financial and human resources to investigate the natural resources of *B. citriodora*, in order to effectively utilize these populations to provide sufficient genetic diversity for continuing improvement;

- Promote research in ecology, reproductive biology and cultivation technologies of *B. citriodora*. The priorities should be focused on environmental and management requirements for commercial plantations, mechanisms of sexual and asexual propagation, reproductive physiology and glasshouse techniques;

- Maintain regular assessment of progeny tests to establish a more comprehensive suite of parameter estimates, in order to improve breeding decisions over the productive life of commercial plantations;

- Further develop the methodology of oil analysis, and sampling techniques for biomass assessment.

In conclusion, with ongoing improvement of the genetic information and cultivation technology for *B. citriodora*, progressively improved planting stock will soon become available in the market. The work reported here, whilst only preliminary, has made an important contribution to this objective.

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